Genetic Screen to Identify Novel Potential Regulators of Dorsal Closure in *Drosophila Melanogaster*

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Summary

Dorsal closure of the *Drosophila* embryo represents the best characterized example of epithelial movement and fusion. This is due to the advanced genetics which allowed the identification of numerous genes that participate in dorsal closure. A deeper knowledge of the molecular mechanisms that drive dorsal closure leads also to a better understanding of other similar epithelial closure events such as wound healing and other processes that involve cell shape changes in general.

The small Rho GTPases Drac1 and Dcdc42 are essential regulators of actin cytoskeleton dynamics and therefore are also key players in dorsal closure. To identify novel signaling components of Drac1 and Dcdc42 that potentially act during dorsal closure, we performed a genetic screen. To this end, we misexpressed dominant negative forms of either Drac1 or Dcdc42 ($Drac1^{N17}$ or $Dcdc42^{N17}$) specifically in the eye by using the Gal4/UAS-system. As a consequence, the resulting tester flies displayed eyes with a disrupted structure ("rough eye" phenotype) that was used as a starting point for a gain-of-function screen. Approximately 10'000 flies with random insertions of a UAS-containing enhancer promoter (EP) element were crossed to the $Drac1^{N17}$ or $Dcdc42^{N17}$ tester flies and the F1 generation was screened for modification of the rough eye phenotype. We selected modifiers that improved eye structure, i.e. suppressed the rough eye phenotype. Modification of the rough eye phenotype is supposed to be caused by EP dependent transcription of a nearby gene. These genes thus encode potential Drac or Dcdc42 signaling components.

The decision to screen for eye (instead of dorsal closure) phenotype modifications was motivated, first, by the viability of the corresponding tester lines that made it possible to screen the F1 generation and, second, by the relative ease to examine the subtle eye phenotype modifications – to score for a partial suppression of the severe dorsal closure phenotypes caused by $Drac1^{N17}$ or $Dcdc42^{N17}$ embryonic overexpression was supposed to be too demanding and time consuming for this kind of large scale screening.

This approach led to the identification of 17 genes, none of which has been previously reported to interact with Drac1 or Dcdc42. Their potential role in dorsal closure has not yet been investigated except for one suppressor gene: *falafel*. Loss-of-function mutations in *falafel* were generated that indeed resulted in various defects in dorsal closure besides other phenotypes. Thus this genetic approach identified at least one novel gene implicated in dorsal closure that probably would not have been picked up in conventional loss-of-function screens as its mutant phenotype is pleiotropic.

falafel encodes a novel RanBP domain containing protein that is evolutionary conserved. Mutations in orthologous genes in *Caenorhabditis elegans* and yeast were described to result in stress hypersensitivity. Furthermore, the yeast and human Falafel orthologs function in a conserved protein phosphatase 4 (PPP4) complex, which, at least in yeast, is sensitive to the anticancer agent cisplatin. In the course of this work, we could also demonstrate that loss of *falafel* function resulted in hypersensitivity to cisplatin, indicating a role in DNA damage response.

Altogether, the 17 identified suppressor mutations represent a heterogenous group of genes with diverse functions. I suggest a common function for six of the 17 genes in mediating Rac/Cdc42 dependent regulation of homeostasis of reactive oxygen species (ROS). This may be relevant, since Cdc42 and especially Rac have well documented roles in the regulation of NADPH oxidase that produces ROS. The six genes with a proposed role in ROS signaling are *Trf2*, *ATP7*, *CG6700*, *CG30421*, *pfrx* and *SelD*.

ROS mediated DNA damage leads to cell cycle arrest. Interestingly, two identified suppressors of Dcdc42^{N17} correspond to well known cell cycle regulators: *string* (cdc25) and *Rbf*. This finding additionally supports a role for Dcdc42 in cell cycle checkpoint regulation in response to ROS. Moreover, Falafel's putative function in DNA damage response may also be in line with the herein proposed new function of Drac1 and Dcdc42 in the regulation of ROS, response to DNA damage and cell cycle progression.

Zusammenfassung

Die Schliessung der dorsalen Epidermis (*dorsal closure)* während der Embryonalentwicklung von *Drosophila* ist eines der best untersuchten Beispiele der Bewegung und Fusion von Epithel-Zellschichten. Dank der fortgeschrittenen *Drosophila*-Genetik wurden bereits zahlreiche Gene identifiziert, welche eine Rolle bei der *dorsal closure* spielen. Die weitere Erforschung der molekularen Mechanismen, welche diesen Prozess steuern, kann auch zu einem besseren Verständnis von ähnlichen Epithelschliessungs-Prozessen wie der Wundheilung oder von anderen Zellbewegungsabläufen führen.

Die kleinen Rho GTPasen Drac1 und Dcdc42 sind essentielle Regulatoren des Actin-Zellskeletts und damit auch wichtig für die Zellbewegungen, die während der *dorsal closure* geschehen. Um neue Komponenten der Drac1- und Dcdc42-Signalwege zu identifizieren, haben wir darum einen genetischen "Screen" durchgeführt.

Zu diesem Zweck exprimierten wir dominant negative Versionen der Drac1- oder Dcdc42-Proteine ($Drac1^{N17}$ oder $Dcdc42^{N17}$) spezifisch im Auge mittels des Gal4/UAS-Systems. Die darausfolgenden "Tester"-Fliegen haben einen "rauhe-Augen-Phänotyp", welcher durch eine unregelmässige Anordnung und Verflachung der Ommatidien gekennzeichnet war. Dieser Phänotyp wurde in der Folge als Ausgangspunkt für einen Überexpressions-Screen benutzt. Über 10000 Fliegen mit zufälligen Insertionen eines "*enhancer promoter*" (EP) Elementes wurden mit den $Drac1^{N17}$- or $Dcdc42^{N17}$-Tester-Fliegen gekreuzt. Daraufhin wurden die F1-Nachkommen auf eine Veränderung des rauhe-Augen-Phänotyps hin untersucht. Es wurden diejenigen Fliegen ausgewählt, welche eine Verbesserung der Augenstruktur, d.h. eine Unterdrückung (*Suppression*) des rauhe-Augen-Phänotyps, aufwiesen. Es wurde jeweils angenommen, dass solch eine Verbesserung der Augenstruktur durch die vom EP-Element und von den darin enthaltenen UAS-Sequenzen abhängige Transkription eines der Insertion angrenzenden Genes verursacht wurde. Solche Gene kodieren darum für potentielle neue Komponenten der Drac1- und Dcdc42-Signalwege.

Der Grund für einen Augen- (statt *dorsal closure*-) Screen lag einerseits in der Lebensfähigkeit der $Drac1^{N17}$- und $Dcdc42^{N17}$-Tester-Fliegen, welche eine Untersuchung schon der F1-Generation möglich machte, und andererseits in der Schwierigkeit, subtile Veränderungen eines schweren *dorsal closure* Defektes in grossem Massstab zu untersuchen.

Im Laufe des Screens wurden 17 Gene identifiziert, welche bisher nicht im Zusammenhang mit Drac1 oder Dcdc42 beschrieben wurden. Die potentielle Funktion im Prozess der *dorsal closure* wurde bisher für eines der 17 Gene untersucht: *falafel.* Wir erzeugten Mutationen im *falafel*-Gen, welche, nebst anderen Phänotypen, tatsächlich eine defekte *dorsal closure* zur Folge hatten.

Somit war dieser genetische Ansatz, ein Überexpressions-Screen für Augenphänotyp-Veränderungen, in mindestens einem Fall erfolgreich, um neue *dorsal closure* Gene zu identifizieren, welche in konventionellen *loss-of-function*-Screens möglicherweise nicht entdeckt worden wären.

falafel kodiert für ein neuartiges, evolutiv stark konserviertes Protein mit einer Ran-bindenden Domäne (RanBP). Verschiedene Studien konnten zeigen, dass orthologe Gene in Fadenwurm, Hefe und Mensch sensitiv für verschiedene Stressbedingungen sind. Ausserdem wurden die menschlichen und die in der Hefe orthologen Proteine als Bestandteil eines Protein-Phosphatase-4 (PPP4) Komplexes beschrieben, welcher sensitiv für das krebshemmende Medikament Cisplatin ist. In dieser Arbeit konnten wir zeigen, dass auch Funktionsverlust von *falafel* zu Hypersensivität für Cisplatin führt. Da Cisplatin ein DNA-schädigendes Molekül ist, zeigen diese Resultate, dass Falafel und der PPP4-Komplex eine Rolle bei Prozessen spielen könnten, die in Folge von DNA-Schädigungen geschehen.

Die 17 in dieser Arbeit identifizierten Suppressor-Gene stellen eine heterogene Gruppe von Genen dar, welche verschiedenste Funktionen ausüben. Für sechs dieser 17 Gene gibt es allerdings Hinweise für eine gemeinsame Funktion, und zwar bei der Regulation von Sauerstoff-Radikalen (*reactive oxygen species, ROS*), bzw. von deren Konzentration und Signalwirkungen in der Zelle. Diese sechs Gene sind *Trf2*, *ATP7*, *CG6700*, *CG30421*, *pfrx* und *SelD*. Interessanterweise haben Cdc42 und vor allem Rac eine gut dokumentierte Funktion in der Regulation der NADPH-Oxidase, einem Enzym-Komplex, welches Sauerstoffradikale produziert. Sauerstoff-Radikale können auch die DNA schädigen, welches in der Folge zu einem Zellzyklus-Stopp führen kann. Interessanterweise entsprechen zwei der identifizierten Dcdc42^{N17}-Suppressoren bekannten Regulatoren des Zellzyklus: *string* (cdc25) und *Rbf*. Die Identifizierung dieser zwei Zellzyklus-Regulatoren, der sechs potentiellen *ROS*-Regulatoren und von Falafel als Interaktoren von Drac1 und Dcdc42 legt somit folgende zusätzliche Funktionen dieser zwei Rho GTPasen nahe: Regulation des Zellzyklus und die Koordination der Antworten (Reparatur bis Zelltod) auf DNA-Schädigungen, möglicherweise in Abhängigkeit von Sauerstoff-Radikalen.

1. Introduction

1.1. Dorsal closure

Dorsal closure (DC) is the morphogenetic movement at the end of *Drosophila* embryogenesis whereby a naturally occurring epithelial hole on the dorsal side gets closed. This hole closure process requires cytoskeletal reorganization, cell shape changes, cell migration, supracellular contraction of entire tissues, and adhesion guided by filopodia and lamellipodia. This process shows obvious parallels to other morphogenetic processes such as embryonic wound healing in *Drosophila* and vertebrates (Grose and Martin, 1999; Stramer et al., 2005), thorax closure in *Drosophila* (Martin-Blanco et al., 2000), neural tube closure in vertebrates (Xia and Karin, 2004), ventral enclosure in *Caenorhabditis elegans* (Simske and Hardin, 2001) and epiboly in zebrafish (Koppen et al., 2006). Moreover, there appears to be also a significant conservation of the molecular mechanisms, since many of the proteins important for DC are also implicated in other epithelial closure events (see below).

Drosophila DC represents the best characterized example of epithelial movement and fusion. This is due to the advanced genetics which allowed the identification of more than 30 genes that participate in DC (Harden, 2002). Live imaging of embryos expressing GFP-fusion proteins further unraveled the dynamic behaviour of cells and their cytoskeleton during the process (Edwards et al., 1997; Jacinto et al., 2000). All this makes *Drosophila* DC an excellent model system in which to study the regulation of cell migration and cell shape changes.

In the *Drosophila* embryo, gastrulation is followed by retraction of the germ band, which leaves the dorsal-most portion of the embryo covered by the large, flat cells of the amnioserosa (Fig. 1). Within approximately five hours the amnioserosa sinks internally where it undergoes apoptosis while the two migrating flanks of the epidermis move towards the dorsal midline, completely sealing the hole 13 hours after egg laying (AEL), by stage 15 (Campos and Hartenstein, 1985). Movement of these epithelial layers depends exclusively on cell shape changes and does not require cell division or cell recruitment (Martinez Arias, 1993).

The dynamics of dorsal closure can be visualized in living embryos with the use of green fluorescent protein (GFP) transgenes that label the actin cytoskeleton (Fig. 1).

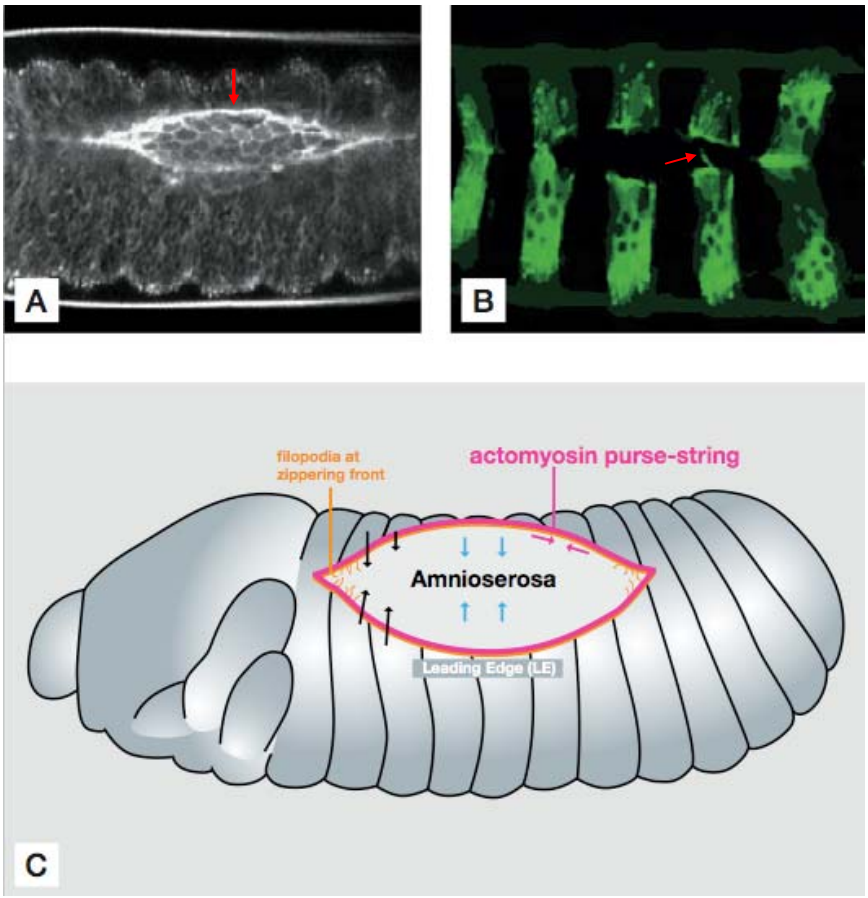


Figure 1. Dynamics of DC can be followed in live embryos expressing GFP-tagged proteins
Imaging of live embryos expressing either GFP-tagged Moesin, which binds cortical F-actin, (A) or GFP-tagged actin (B) reveals a thick actin cable and dynamic filopodia (arrows) at the leading edge (LE). Upon heat shock a temporary ubiquitous expression of hs-GFP::Moesin is obtained, shown in (A). A more restricted expression of UAS-GFP::actin is obtained with the enGal4 driver (B). In this situation the very thin and dynamic filopodia become visible above non fluorescent dark regions, especially the amnioserosa. In (C) a schematic embryo around stage 13/14 is shown. The contributions of different forces that drive DC are indicated by arrows (cell elongation of lateral epithelia, black arrows, and amnioserosa contraction, blue arrows) and by the red actomyosin purse-string (red arrows) and filopodia at the LE.

The entire process has been divided into four overlapping phases (Jacinto et al., 2002b):

1) Initiation of dorsal closure starts prior to the completion of germ band retraction, at stage 12, 8 hours after egg laying (AEL), as soon as the anterior region of amnioserosa and adjacent leading edge (LE) has been revealed by the moving germ band. At this first stage, hole closure is rather slow and seems to involve mainly amnioserosa cell contraction leading to movement of the attached leading edge cells (Kiehart et al., 2000).

2) At stage 13, 9 hours AEL, the lateral epithelial cells start to elongate dorsally (Jacinto et al., 2002b; Riesgo-Escovar and Hafen, 1997b). Furthermore, the first row of cells at the front accumulate at the dorsal edge a thick bundle of F-actin and non-muscle myosin heavy chain II (hereafter referred to as myosin) which seems to form a supracellular contractile cable around the entire dorsal hole (Jacinto et al., 2002a; Young et al., 1993). This actomyosin cable potentially operates like a “purse-string” to draw the epithelial hole closed (Kiehart, 1999; Young et al., 1993). The cable also seems to organize the scalloped epithelial edge into the uniform advance front, thereby preventing individual cells from migrating forward on their own (Jacinto et al., 2002a). The cable seems to be anchored by E-cadherin-mediated adherens junctions at the LE, thereby joining the cells to form a contiguous purse-string (Danjo and Gipson, 1998).

3) Dynamic filopodial and lamellipodial protrusions of the leading edge cells are characteristic for the third phase of dorsal closure, starting at stage 14, 11 hours AEL. These actin protrusions extend from each LE to actively zipper the epithelial sheets together, i.e., when the opposing LE cells meet at the dorsal midline, these filopodia interdigitate and prime the formation of adherens junctions (Jacinto et al., 2000) as it happens in cultured keratinocytes that assemble into an organized epithelial sheet (Vasioukhin et al., 2000).

Filopodia also seem to support the correct matching of the opposing embryonic segments since specific mutants that lack filopodia frequently display mismatched segments (Jacinto et al., 2000). A sensing mechanism analogous to that of axon growth cone filopodia has been postulated. The molecular nature of this mechanism is unknown however. During ventral enclosure in *C. elegans*, the axonal repellent Semaphorin-2A is required to prevent ectopic contacts between migrating cells (Roy et al., 2000). Thus it has been suggested that similarly during DC a repellent could be expressed from some cells of the LE, possibly by the segment border cells (see below), thereby restricting filopodia-guided migration of the LE cells into discrete paths.

4) During the last phase of dorsal closure, at stage 15, 13 hours AEL, the opposing epithelia meet at the midline and stop to migrate. The transient adhesions that have been formed between interdigitating filopodial protrusions convert into tight and permanent adherens junctions. The cells of the amnioserosa are then completely internalized and undergo apoptosis. In the end, there is an invisible midline and a completely sealed hole.

In summary, DC seems to be driven mainly by contraction of both the amnioserosa and the supracellular „purse string“. Filopodia and lamellipodia are required to zip the opposing leading edges together. A recent report also implicates, in addition to the amnioserosa, a second extraembryonic tissue, the yolk sac, to contribute to DC (Reed et al., 2004).

1.2. Genes involved in dorsal closure

Genes that affect dorsal closure can be grouped into at least five classes and are summarized in Table 1 (reviewed by Harden 2002). Mutations in genes involved in either the Jun amino-terminal kinase (JNK) cascade, the transforming growth factor beta (TGF-ß) signaling pathway or the Wingless (Wg) signaling pathway can disrupt dorsal closure. Furthermore genes involved in the Rho GTPase-mediated signaling pathway, and genes encoding cytoskeletal proteins and membrane-associated molecules involved in cell adhesion are required for DC.

Some dorsal closure genes do not fit into one of the five classes and are not described here. Only the below mentioned genes are listed in Table 1. For example, Ras signaling will not be discussed, neither extracellular proteins as type IV collagen that have been shown to play a role during dorsal closure. For a more complete picture of dorsal closure genes see reviews by Harden (2002) and Martin and Parkhurst (2004).

DC gene classes	DC mutants	role in DC
JNK (chapter 1.2.1.)	*msn, slpr, hep, bsk, DJun, DFos (kayak)*	initial signal in LE, regulation of actin/myosin cytoskeleton, required for filopodia formation
TGF-ß (1.2.2.)	*dpp, put, tkv, mad, shn, pnr*	expansion of signal, regulation of cytoskeleton at segment border cells
Wg (1.2.3.)	*wg, arm* (ß-catenin)*, dTCF*	regulation of JNK, link to adherens junction?
cytoskeletal and membrane-associated proteins (1.2.3.)	*scb, mys* (integrins); *actin*, *myosin; arm* (ß-catenin), *shg* (E-cadherin); *abl, ena*	cytoskeleton and signaling
Rho GTPase family (1.2.4.)	*RhoA, Drac1/2, mtl, Dcdc42*	regulation of cytoskeleton dynamics and of gene expression

Table 1. Dorsal closure genes classified into five groups

1.2.1. JNK

Coincident with the first cell shape changes of the lateral epidermis, there is an upregulation of the JNK pathway in the LE cells whereas in the opposing amnioserosa JNK activity is downregulated. This boundary of JNK activity is established in response to the early dorsoventral patterning system, involving the Dpp gradient, and subsequent inductive signaling between the amnnioserosa and the adjacent epidermis (Reed et al., 2001; Stronach and Perrimon, 2001).

JNK cascades consist of a set of sequentially activated serine/threonine kinases closely related to the extracellular regulated protein kinases (ERK) that transmit cytoplasmic signals to the

nucleus (Goberdhan and Wilson, 1998). In mammalian cells, JNKs are regulated by various cellular stresses and growth factors and have been implicated in the regulation of many diverse biological processes, such as cell shape changes, immune responses and apoptosis (Davis, 2000).

In *Drosophila* these kinases are encoded by the *slipper*/DJNKKK (*slpr*) (Stronach and Perrimon, 2002), *hemipterous*/DJNKK (*hep*) (Glise et al., 1995) and *basket*/DJNK (*bsk*) (Riesgo-Escovar et al., 1996; Sluss et al., 1996) genes. A Ste20-related kinase, Misshapen (Msn), is the putative JNKKKK (Davis, 2000; Su et al., 1998) and functions upstream of Slpr, Hep and Bsk in the dorsal closure process. The transcription factor Djun is activated by DJNK (Hou et al., 1997; Kockel et al., 1997; Riesgo-Escovar and Hafen, 1997a). In addition to Djun, two other transcription factors have been identified that act in the JNK pathway during DC —the *Drosophila* homolog of Fos, DFos (Riesgo-Escovar and Hafen, 1997a; Zeitlinger et al., 1997), and the ETS-domain protein anterior open Aop/Yan (Riesgo-Escovar and Hafen, 1997b). Both DJun and DFos act as positive regulators, probably through the formation of a heterodimer termed AP-1, whereas Aop functions as a negative regulator in the process.

Mutations in any of these JNK pathways components result in DC defects. The embryos eventually die with a characteristic dorsal open phenotype which is easily identified in cuticle preparations (compare to Fig. 5 C and E, Chap. 2.2.2.) (Martinez Arias, 1993; Nusslein-Volhard et al., 1985; Perrimon et al., 1989; Wieschaus et al., 1984).

A more detailed analysis revealed that loss of JNK signaling is characterized by disruption of the actin and myosin cytoskeleton at the LE, subsequent failure of cell elongation and missing filopodia (Jacinto et al., 2000).

The transcriptional targets of JNK include at least one cytoskeletal component, encoded by *chickadee* (Jasper et al., 2001). Chickadee is the *Drosophila* homolog of Profilin which regulates actin assembly, and there is genetic evidence for an essential role of *chickadee* in DC (Jasper et al., 2001). Furthermore, the dual-specific phosphatase encoded by *puckered* is activated by the JNK cascade in the leading edge cells (see below Fig. 2, *puc*-lacZ enhancer trap line) and acts in a negative feedback loop by dephosphorylating and thereby inactivating Bsk (Martin-Blanco et al., 1998). *puckered* mutant embryos have thus elevated JNK activity. Intriguingly, this leads to a somewhat unrestricted movement and overcontraction of the advancing epidermis.

Furthermore, expression of scab and myospheroid, two *Drosophila* integrins, have been shown to be activated by JNK signaling (Homsy et al., 2005).

A recent review on the role of the JNK pathway in dorsal closure is given by Xia and Karin (2004).

1.2.2. Dpp/TGF-ß

Another important output of JNK signaling is the induction of Decapentaplegic (Dpp, a TGF-ß homolog) expression in the LE cells (Glise and Noselli, 1997; Hou et al., 1997; Jackson and Hoffmann, 1994; Riesgo-Escovar and Hafen, 1997b; Sluss and Davis, 1997; St Johnston and Gelbart, 1987; Stronach and Perrimon, 2002; Zeitlinger et al., 1997). Involvement of a TGF-ß signaling pathway operating during DC was demonstrated by showing that mutations in the genes *punt* (*put*) (Letsou et al., 1995; Ruberte et al., 1995) and *thick veins* (*tkv*) (Affolter et al., 1994; Brummel et al., 1994; Nellen et al., 1994; Penton et al., 1994), which encode type-II and type-I TGF-ß receptors respectively, cause DC defects. Furthermore, overexpression of both Dpp or an activated form of the Dpp receptor Tkv rescues substantially the DC defects of JNK pathway mutants (Chen et al., 2002; Hou et al., 1997; Riesgo-Escovar and Hafen, 1997b; Sluss and Davis, 1997; Su et al., 1998). Mothers against Dpp (Mad), a *Drosophila* Smad, signals downstream of Dpp to induce gene transcription. Embryos deficient in Mad show DC defects. Two other transcription factors that are also required for DC are Schnurri (Shn) that has been shown to interact directly with Mad in a Dpp-dependent manner (Udagawa et al., 2000), and Pannier (Pnr) (Calleja et al., 2000; Herranz and Morata, 2001). One of the target genes of Dpp signaling in the LE is *zipper* (*zip*), which encodes the heavy chain of non-muscle myosin II (Arquier et al., 2001).

The DC defects of Dpp pathway mutant embryos are distinct to the JNK pathway mutant phenotype. Despite the lack of myosin synthesis at the LE in *tkv* mutant embryos, assembly of the actomyosin cytoskeleton does not require functional Dpp signaling (Ricos et al., 1999; Riesgo-Escovar and Hafen, 1997a). This can probably be explained by sufficient maternally produced myosin (Young et al., 1993).

The exact role of Dpp signaling in DC is not clear yet. It was proposed that while Drac1 (see below) and JNK signaling assemble cytoskeletal components and other proteins (Dpp, Puc, and DPAK) in the LE cells to initiate cellular migration, Dpp-activated signaling would control the dynamics of epidermal migration, via Dcdc42 (see below) (Ricos et al., 1999). Dpp secretion from already elongating LE cells possibly triggers more laterally located cells to adopt the same elongation behaviour. Another role of Dpp and Dcdc42 signaling in regulating the LE cytoskeleton specifically at the segment borders is discussed below.

Thus, JNK activity leads to transcription of further regulators of DC and of cytoskeletal components that are required for the cell shape changes occurring during DC. However, there seems to be a transcription-independent role for some components of the JNK pathway. For example, Bsk/DJNK possibly regulates cytoskeleton dynamics through direct interaction with

p150-Spir that belongs to the Wiscott-Aldrich syndrome protein (WASP) homology domain 2 (WH2) family of proteins involved in actin reorganization (Otto et al., 2000).

1.2.3. JNK regulation, cytoskeleton and adherens junction

There are several other proteins that also induce transcription of *dpp* and *puc* at the LE, either by activating the JNK cascade or through another JNK independent route. Thus *dpp* and *puc* are widely used to measure JNK activity either by doing *in situ* hybridization of *dpp* transcripts or by assaying β-galactosidase activity from a lacZ enhancer trap insertion in *puc* (Ring and Martinez, 1993) (Fig. 2).

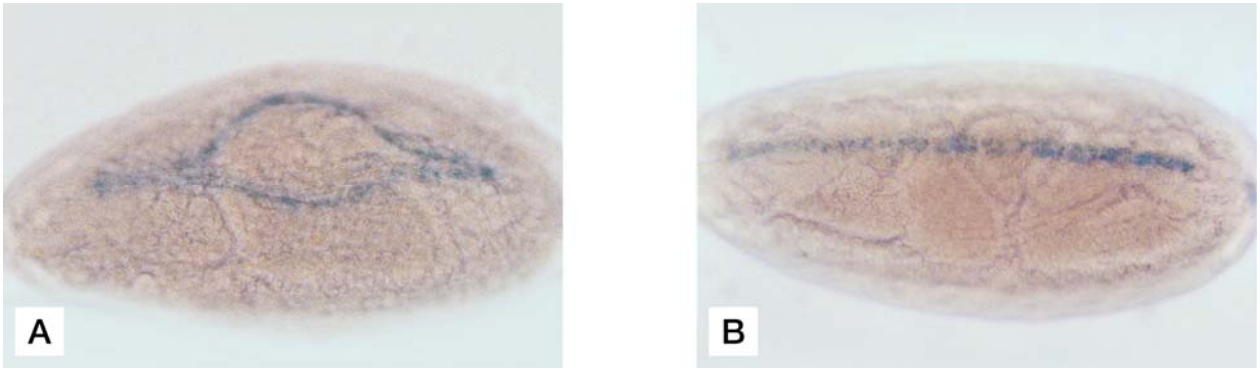


Figure 2. Levels of *puckered*-lacZ expression in the leading edge cells as indicator for JNK activity
puc-lacZ expression during (A) and at the end (B) of dorsal closure. The advancing epidermis is stained only in the leading edge (LE) cells which eventually form the dorsal midline (B)

Mutations in genes that lead to altered *dpp* or *puc* expression at the LE often show DC phenotypes. For example, mutations in the non-receptor tyrosine kinases Src42A, Tec29 and Shark show reduced *dpp* and *puc* transcription at the LE and various DC defects (Tateno et al., 2000).

Good candidates to act further upstream are integrins. Indeed, two *Drosophila* integrins, *scab* and *myospheroid*, encoding α and β subunits, respectively, show dorsal holes when mutated (Hutson et al., 2003; Narasimha and Brown, 2004; Stark et al., 1997). The view that integrins serve only as passive mechanical links between exracellular matrix (ECM) molecules and the cell membrane is out of date. Integrin signaling mediates cell adhesion, migration, and invasion during development, tissue repair, tumor invasion, and metastasis. However, it is unlikely that integrins signal through exclusive, integrin-specific pathways. Instead, they appear to cooperate with other cell-surface receptors to influence a variety of signaling pathways (Porter and Hogg,

1998). Homsy et al. recently suggested that *scab* and *myospheroid* rather act downstream of JNK signaling (Homsy et al., 2005). Notch signaling appears to inhibit JNK mediated transcription of *dpp* and *puc*, and complete loss of Notch also results in DC defects. Furthermore, two adherens junction proteins, Canoe and ZO1 are required for dorsal closure, also by activating the JNK pathway. Thus, besides the proposed function for adherens junctions in anchoring the actin-myosin cable, they also appear to assemble signaling complexes that regulate DC.

Enabled (Ena), a modulator of actin dynamics, localizes to adherens junctions and is regulated by the nonreceptor tyrosine kinase Abelson (Abl). *abl* and *ena* mutant embryos exhibit defects in several morphogenetic processes including DC. The effect on DC is further enhanced by mutations in two other genes encoding adherens junction proteins, *armadillo (arm)* and *shotgun (shg)*, the *Drosophila* homologs of β-catenin and E-cadherin, respectively (Grevengoed et al., 2001). The requirement for Arm during DC has been reported previously (McEwen et al., 2000). Arm binds α-catenin and E-cadherin, and regulates assembly of F-actin at adherens junctions (Grevengoed et al., 2001; Takahashi et al., 2005). Arm has a second well defined role in Wingless (Wg) signaling where it localizes to the nucleus to activate gene transcription (DasGupta et al., 2005). Intriguingly, Wingless itself as well as Dsh, a Wg signaling component, are also required to activate *dpp* and *puc* expression via the JNK pathway and to coordinate cell shape changes during dorsal closure (McEwen et al., 2000). However, considering the stronger DC phenotype of *arm* compared to *Wg* mutant embryos, Arm may contribute to DC in a Wg-independent fashion through its role in adherens junctions (McEwen et al., 2000).

1.2.4. Rho GTPases

Small GTPases of the Rho subfamily have been shown in various systems to be key regulators of the actin cytoskeleton and upstream activators of JNK cascades (Bishop and Hall, 2000). Thus, they are good candidates to control the cytoskeletal re-arrangements required for the cell shape changes that occur during DC. Indeed, five of the six *Drosophila* Rho GTPases are involved in DC: the Drosphila Rac proteins Drac1, Drac2, and Mtl, the *Drosophila* Cdc42 protein Dcdc42, and the *Drosophila* Rho protein RhoA (also called Rho1). The particular functions of each Rho GTPase for DC are not completely understood yet, but they seem to have only partially overlapping functions and most likely regulate different aspects of DC. These include the regulation of the actin/myosin cytoskeleton at the LE, formation of signaling complexes and assembly of F-actin at adherens junctions, formation of filopodia and lamellipodia, as well as regulation of the JNK and Dpp pathways. A sequential activation of Dcdc42, Drac1 and RhoA

has been described in fibroblasts (Nobes and Hall, 1995) and macrophages (Allen et al., 1997). However, they appear to function in parallel during DC (Harden et al., 1999).

Whether any of the *Drosophila* Rho GTPases regulates JNK signaling during DC remains a controversial issue. Several studies using dominant negative and constitutively active transgenes implicated Drac1 and Dcdc42 as operating upstream of JNK signaling in DC (Glise and Noselli, 1997; Harden et al., 1996; Harden et al., 1995; Harden et al., 1999; Stronach and Perrimon, 2002). For example, expression of constitutively active DJun can partially rescue the DC defects induced by dominant negative Drac1 (Hou et al., 1997).

On the other hand, embryos expressing dominant negative versions of Drac1 ($Drac1^{N17}$) or Dcdc42 ($Dcdc42^{N17}$), or a negative regulator of Drac1, RotundRacGAP, maintain *dpp* and *puc-lacZ* expression at the LE (Raymond et al., 2001). The same is true for Dcdc42 mutant embryos (Genova et al., 2000). However, a recent study investigated the Drac1/Drac2/mtl triple mutant embryos with respect to JNK activity (Woolner et al., 2005). In these embryos *dpp* expression at the LE is reduced and DFos was no longer localized to the nucleus, indicating that JNK signaling is indeed regulated by Drac. Moreover, expression of a constitutively active form of JNKK, *hemipterous* (*UAS-hep*^{CA}), partially rescues the Drac1 mutant phenotype (Woolner et al., 2005).

Ricos et al. (1999) showed that embryos mutant for Dpp pathway genes resembled the embryos in which $Dcdc42^{N17}$ is overexpressed. In these embryos, DPAK is lost in the LE cells, which are subsequently pulled towards the segment borders, thereby causing a bunching of the epidermis. This bunching phenotype is probably due to excessive contraction of the LE only at the segment borders. The serine/threonine kinase DPAK has been suggested to prevent these overcontractions by locally loosening the actin cytoskeleton (Harden et al., 1996; Ricos et al., 1999).

$Drac1^{N17}$ overexpression leads to a similar phenotype as JNK pathway mutants that is clearly distinct from the $Dcdc42^{N17}$/Dpp phenotype. $Drac1^{N17}$ overexpression disrupts formation of the leading edge actomyosin purse-string and impairs cell elongation (Harden et al., 1995; Harden et al., 1999) as it happens in JNK pathway mutant embryos. Analysis of the triple Rac mutant embryos confirm these results (Hakeda-Suzuki et al., 2002). In addition, there is a gross reduction of filopodia and lamellipodia at the LE. Live imaging demonstrated that these actin protrusions were also missing in *hep* mutant embryos and the resulting phenotype includes a severe misalignment of opposite segments along the zippering seam (Jacinto et al., 2000). Altogether, these data suggest that Rac signaling indeed functions upstream of the JNK cascade.

Cdc42 has been implicated in the regulation of filopodia (Nobes and Hall, 1995), and this seems to be the case also during DC, since expression of $Dcdc42^{N17}$ blocks filopodia formation leading

to a segmental misalignment phenotype similar to that of *hep* mutant embryos (Jacinto et al., 2000). Cell culture studies implicated the Wiskott-Aldrich protein (WASP), profilin and the Arp2/3 complex downstream of Cdc42 to mediate *de novo* actin polymerization in filopodia (Takenawa and Miki, 2001). *Drosophila* WASP, however, does not seem to be essential for DC (Ben-Yaacov et al., 2001; Tal et al., 2002). Dcdc42 (or Drac) may still act through profilin in driving filopodia formation, as mutations in the *Drosophila* profilin, *chicadee*, lead to loss of filopodia at the LE (Jasper et al., 2001).

However, the role of Dcdc42 in DC remains ambiguous. The simplest interpretation puts Dcdc42 downstream of Dpp. This is supported, first, by the above mentioned phenotypic similarity of Dpp pathway mutant and $Dcdc42^{N17}$ expressing embryos, and second, by the ability of a constitutively active form of Dcdc42 to partially rescue the DC phenotype of a mutation in *thick veins*, which encodes the Dpp receptor (Ricos et al., 1999).

RhoA is also important for DC. It appears to be required for the contractility of the purse-string. Embryos expressing a dominant negative form of RhoA and embryos mutant for RhoA show loss of myosin from the LE (Harden et al., 1999; Magie et al., 1999). Furthermore, *zipper*, encoding the *Drosophila* non muscle myosin-II heavy chain, interacts genetically with RhoA (Halsell et al., 2000), *Drosophila* Rho-associated kinase (Drok) and the myosin-binding subunit (DMBS) of myosin phosphatase (Mizuno et al., 2002). Drok overexpression as well as mutations in DMBS result in excessive phosphorylation of the myosin regulatory light chain (MRLC) and aberrant cable assembly at the LE, leading to failure in DC (Mizuno et al., 2002).

A closer look at RhoA mutant embryos reveals that loss of myosin occurs only at the segment boundaries. Therefore the cells with normal myosin levels show anterior-posterior contraction, while the segment border cells fail to contract and tend to splay out (Harden et al., 1999; Magie et al., 1999). The overall effect is an uneven constriction of the LE along the A/P axis.

Recent evidence suggests that this phenotype may not only be a consequence of unevenly distributed myosin but also a result of an altered adhesive property of RhoA mutant cells. That is, cells at the segment borders form ectopic contacts with their lateral neighbors (Bloor and Kiehart, 2002; Magie et al., 1999). A role for the Rho and Rac small GTPases in cadherin-dependent cell-cell contact formation has been reported for cultured vertebrate cells (Braga, 2000; Braga et al., 1997). Interestingly, *Drosophila* RhoA accumulates at sites of cadherin based adherens junctions at the LE, and DE-cadherin and α-catenin localization is disrupted in RhoA mutant embryos (Magie et al., 2002). Moreover, RhoA binds directly to α-catenin and to p120catenin ($p120^{ctn}$) *in vitro* (Magie et al., 2002). $p120^{ctn}$ is suggested to recruit RhoA to sites of cadherin based adherens junctions. Thus the DC defects of RhoA mutants could also be the result of aberrant adherens junction formation. Rho signaling in response to the above

mentioned predicted repellent could thereby induce differential adhesion properties of cell populations. In this model, segment border cells are likely candidates to express different adhesion molecules that would not be “recognized” by filopodia of cells inbetween the segment borders.
It is not clear whether RhoA is required for activation of the JNK pathway. RhoA mutant embryos exhibit normal levels of *dpp* mRNA and *puckered-lacZ* expression in the LE cells (Magie et al., 1999). Embryos expressing dominant negative RhoA, however, show some ectopic activation of JNK dependent transcription in the lateral epidermis (Bloor and Kiehart, 2002).

In summary, the LE cells are induced by signaling in the adjacent amnioserosa to activate the JNK pathway and to accumulate F-actin and myosin at their dorsal end. Adherens junctions are important in both anchoring the actomyosin cable and assembling signaling complexes that regulate cytoskeletal rearrangements as well as JNK dependent transcription. The JNK cascade is regulated by numerous proteins, including non-receptor tyrosine kinases, the Rho GTPase Drac1, Notch and members of the Wg pathway. JNK signaling in the LE leads to secretion of Dpp, which regulates DC in one way through Dcdc42 and DPAK. DPAK function is possibly needed to both loosen and strengthen the cytoskeleton depending on the position along the anterior/posterior axis. RhoA seems to regulate both myosin activity and adhesive properties of LE cells. JNK signaling, the three *Drosophila* Racs, Drac1, Drac2 and Mtl, and probably also Dcdc42 are required for the formation of filopodia and lamellipodia. These actin protrusions appear to be responsible for the correct matching of opposing LEs at the dorsal midline, possibly by sensing the RhoA-mediated differential adhesive properties of LE cells.
Figure 3 summarizes the signaling events occurring during DC. Various further proteins that participate in DC have not been mentioned here and are not included in the figure (for a more complete picture of DC proteins see review Harden 2002).

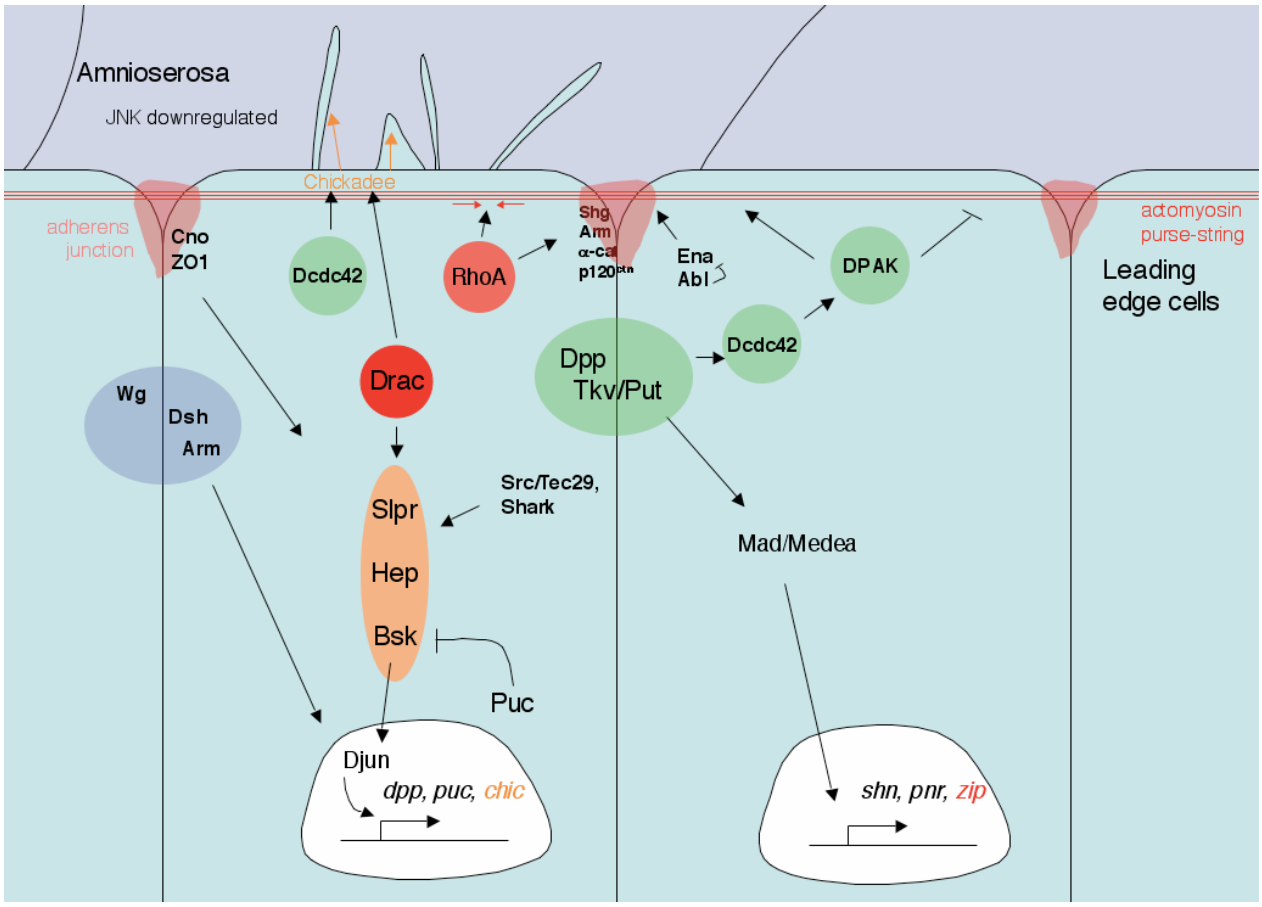


Figure 3. Schematic diagram of signaling events occurring in LE cells.

Central is the JNK cascade, represented by the Slpr, Hep and Bsk proteins, that is regulated in many ways. The effects of subsequent TGF-β/Dpp signaling are less clear but may involve instruction of more ventrally located cells to acquire the same cell shape changes as the LE cells. Rho GTPases regulate various components of the cytoskeleton. In addition Drac1 and possibly the two other related Drac2 and Mtl probably activate the JNK pathway. Adherens junctions anchor the cytoskeleton and assemble signaling complexes.

1.2.5. DC is similar to other morphogenetic processes in both mechanistic and molecular aspects

The spreading and fusion of epithelia, and the accompanying phenomena like polarized F-actin accumulation, supracellular purse-strings, filopodia-mediated adhesion, occur in various morphogenetic processes. Although these processes remain much less well characterized than *Drosophila* DC, it is becoming apparent that they require many of the same proteins participating in DC. For example, wound healing in *Drosophila* also requires JNK signaling (Ramet et al., 2002) and Rho family small GTPase function (Stramer et al., 2005). Wound healing in

vertebrates requires TGF-β signaling and Rho family small GTPase function (Jacinto et al., 2001). During eyelid closure in mammals TGF-β activates the JNK pathway (Zhang et al., 2003a). Closure of the neural tube (Sabapathy et al., 1999) and optic fissure in vertebrates also require JNK (Weston et al., 2003; Xia and Karin, 2004), and hmp2/β-catenin and other adherens junction proteins participate in ventral enclosure in *C. elegans* (Simske and Hardin, 2001).
We have now more than a basic understanding of the cytoskeletal changes and the complex network of signaling pathways that are necessary to promote *Drosophila* DC. This is due to the advanced genetics and the ability to investigate the dynamic cellular behaviours in live embryos. Thus DC appears as an ideal model system for epithelial closure events that occur during normal development and possibly more generally for pathological conditions, where cell migration is misregulated, such as in metastasis.

The aim of this work was to genetically identify novel genes that potentially are involved in DC. A deeper knowledge of the morphologic and molecular mechanisms that drive dorsal closure also leads to a better understanding of other similar epithelial closure events such as wound healing and may as well elucidate the genetics of dynamic cell behaviour in general.
To complement conventional loss-of-function screens we decided to perform a gain-of-function screen. We screened for genes that, upon P-element-mediated overexpression, interacted with Drac1 or Dcdc42. We identified several genes not implicated in dorsal closure so far. However, their role in this process remains to be shown. For one of these genes loss-of-function mutations were generated that indeed show various defects in dorsal closure besides other phenotypes. Further analysis revealed that the gene encodes a subunit of a phosphatase complex.
In addition, the results of this thesis additionally suggest new functions for Rac and Cdc42 signaling in body size control, ROS homeostasis, and DNA damage repair.

2. Genetic screen to identify novel potential regulators of dorsal closure

2.1. Gain-of-function screen for Drac1 and Dcdc42 interactors

In order to identify novel components that are required for DC we have performed a genetic screen. Specific mutations in Drac1 and Dcdc42 were used as a starting point. Drac1 mutants are viable and fertile and display axon guidance and, to a lesser extent, axon branching defects (Ng et al., 2002). Only triple Rac (Drac1, Drac2 and mtl) mutants display dorsal closure defects. Dcdc42 homozygous mutant embryos fail to retract the germband and subsequently die before

the onset of dorsal closure (Genova et al., 2000). We therefore decided to misexpress dominant negative forms of Drac1 and Dcdc42 specifically in the eye to obtain viable flies displaying a clear rough eye phenotype. This phenotype was used as a basis to screen for modifying mutations. The viable tester line allowed us to screen for modifiers already in the first generation (F1 screen). This would not be possible if we wanted to misexpress the dominant negative forms of Drac1 and Dcdc42 in the embryo and to screen for modifiers of the resulting dorsal open (and lethal) phenotype.

Furthermore, in contrast to the conventional loss-of-function screens, we chose to perform a gain-of-function screen. We adapted the enhancer-promoter (EP) screen developed by Rorth (Rorth, 1996; Rorth et al., 1998). Gain-of-function mutations are produced by UAS-containing P-elements that are randomly inserted into the genome. Nearby genes become overexpressed when a Gal4 source is provided. Therefore we screened for genes that, upon overexpression, led to a modification of the rough eye phenotype.

In the following chapter, I will explain in more detail the rationale of our screening strategy and describe the molecular nature of the P-element and the tester lines we used.

2.2. Choosing the screening strategy: EP Screens

In conventional genetic screens, mutations that reduce or eliminate a gene function are identified following random mutagenesis of the genome with chemicals, ionizing radiation or transposable elements. However, it has been estimated that only around 30% of all *Drosophila* genes can be mutated to an easily identifiable phenotype (Ashburner et al., 1999; Miklos and Rubin, 1996). This may be due to the fact that some genes have a pleiotropic phenotype. Another reason may be redundancy, i.e., a gene may be dispensable if the genome contains redundant, back-up copies of the gene or if the product of another, although not homologous, gene can exert and thereby replace the function of the mutated gene. For example, the two homologous genes scylla and charybde act as partially redundant genes in the regulation of growth in *Drosophila* (Reiling and Hafen, 2004). Furthermore, an early lethal phenotype hinders studies of later functions of the respective gene.

Not only loss but also gain of function of a gene can result in specific phenotypes. Genes with missing, mild or pleiotropic loss-of-function phenotypes may therefore be identified based on their gain-of-function phenotypes. Rarely, gain-of-function mutants have been isolated from conventional mutagenesis screens. They could be identified based on their dominant phenotypes. Gain-of-function phenotypes, however, can also be generated by controlled

overexpression of a gene of interest. In addition, controlled overexpression allows to identify important genetic interactions. This is possible if increased expression of one gene enhances or suppresses the phenotype of a mutation in another gene, especially when their products are involved in the same process. For example, overexpression of the *Drosophila* JNK, *bsk,* rescues the dorsal open phenotype of a *cno* mutant embryo (Takahashi et al., 1998). Similarly, overexpression of dominant negative Drac1 leads to defective DC, which is rescued by expression of activated Djun (Hou et al., 1997).
However, phenotypes caused by overexpression must be interpreted with caution. Overexpression of a gene may lead to non-physiological high levels of its product and may eventually produce a non-specific phenotype (see also discussion Chap. 2.8.).

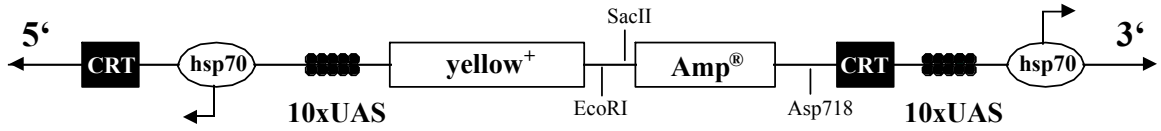
To allow systematic overexpression screens in *Drosophila*, Rørth (1996) developed a modular system combining P-element insertional mutagenesis with Gal4 regulated gene expression. The target P-element carries upstream activating sequences (UAS) that are recognized by the yeast transcription factor Gal4, and a basal promoter oriented to direct the expression of genomic sequences adjacent to the P-element insertion site (Fig. 4 and 6). Rorth named these P-element insertion lines 'EP' to indicate that both the enhancer (Gal4 binding sites) and the promoter are included in the P-element. When combined with a source of Gal4, the P-element will direct expression of any gene that lies next to its insertion site. The fact that P-elements insert preferentially into 5' ends of genes (Spradling et al., 1995) greatly increases the number of essentially full-length transcripts (Rorth, 1996). The modular design makes the screen flexible and conditional, i.e. genes can be induced in any spatial or temporal pattern using different Gal4-lines. This misexpression approach has been successfully adopted in screens that aimed at identifying genes involved in specific developmental processes. In some reports, tissue specific Gal4 driver lines were crossed with EP element lines. The resulting overexpression of genes adjacent to the EP element were screened for specific phenotypes (Abdelilah-Seyfried et al., 2000; Kraut et al., 2001; Pena-Rangel et al., 2002; Rorth, 1996; Tseng and Hariharan, 2002). In other reports, the Gal4 driver lines carry in addition a mutation in a gene of interest causing a specific phenotype. These flies were crossed to the EP element lines and the F1 generation was screened for modifiers of this phenotype (Huang and Rubin, 2000; Raymond et al., 2004; Rorth et al., 1998). An example of the latter approach was recently published by Raymond et al. (2004). They screened EP element lines for modifiers of the rough eye phenotype caused by overexpression of RacGAP(84C). Proteins with established functions in small GTPase signaling were identified as well as novel potential partners of RacGAP(84C), none of which had been found in previous saturating loss-of-function screens (Raymond et al., 2004).

The aim of the present study was to identify novel genes implicated in the process of DC. Loss-of-function mutageneses have already identified many DC genes. Due to the above mentioned limitations of loss-of-function screens we chose to perform a complementary gain-of-function modifier screen. Moreover, the modular design of the EP screen developed by P. Rorth makes it simple to use for the following reasons: many useful Gal4 pattern lines are already available; target lines are easy to generate and can be used repeatedly. As the mutagen is a P-element, the cloning of affected genes is greatly facilitated.

We adapted the screening strategy introduced by Rorth to identify DC genes. To this end, we generated a large number of EP element insertion lines and screened them for modifiers of a specific phenotype caused by mutant regulators of DC (see below).

2.2.1. P-element with bidirectional upstream activator sequence (UAS) enhancers

The EP element used in this study is slightly modified from that described by Rørth (Rorth, 1996; Rorth et al., 1998). The EP element used here (Fig. 4) contains Gal4 binding sites (UAS) and basal promoters (hsp70) at both ends of the P-element. Thus this double-headed element is able to drive the expression of endogenous genes on both sides of the insertion, thereby



potentially doubling the number of genes that can be screened. A part of the EP element, including the UAS site at the 5' end, is flanked by Cre recombinase target (CRT, loxP) sites. This cassette is removed in flies expressing Cre recombinase, so that overexpression of an endogenous gene 5’ to the P element is lost. This allows to distinguish whether the gene 5’ or 3’ to the P element is responsible for the modified phenotype. In total we generated 10000 independent EP element insertions (see Materials and Methods).

Figure 4. Transcription from mobilized P elements was induced from bidirectional upstream activator sequence (UAS) enhancers using GMR-Gal4

The “double-headed” EP element contains UAS sequences at both sides of the P-element. Cre recombinase excises the region between the Cre recombination target (CRT) sequences, including UAS sequences and the hsp70 promoter at the 5' end as well as the *yellow* reporter and the Ampicillin resistance gene (Figure modified from J. Reiling, see also Materials and Methods).

2.2.2. The tester lines GMR>Drac1^{N17} and sev>Dcdc42^{N17}

A large number of independent EP element insertions were generated and tested in a Rac1 or Cdc42 sensitized genetic background.

Drac1 and Dcdc42 are small GTPases that act as molecular switches that cycle between a GDP-bound ‘‘off’’ state and a GTP-bound ‘‘on’’ state. Active GTPases hydrolyze GTP to GDP and thereby get inactive. This GDP is removed by guanine nucleotide exchange factors (GEFs).

We made use of dominant negative forms of Drac1 and Dcdc42 that carry both a T to N amino acid substitution at position 17. This mutation prevents the exchange of a bound GDP to a GTP. Thus, these dominant negative forms are thought to remain in an inactive GDP-bound state that binds to and sequesters endogenous GEFs. The consequent reduction of “free” GEFs results in decreased activation of Drac1, Dcdc42 and probably other RhoGTPases. Thus, it is also conceivable that the Drac1^{N17}/Dcdc42^{N17} overexpression phenotype does not reflect a loss-of-function phenotype of the endogenous proteins but might rather be compared to a composite phenotype of several GEF mutants.

Both Drac1 and Dcdc42 are key regulators of DC. Embryos expressing dominant negative versions of either Drac1 (Drac1^{N17}) or Dcdc42 (Dcdc42^{N17}) die with defects in DC (Fig. 5 C and E). Lethality during embryogenesis prevents the use of these mutants for a screening of the F1 generation, because the tester lines would not survive to adulthood.

Theoretically the methodological difficulties caused by the lethality could be overcome by performing a screen in the F2 generation. To this end, the EP element insertion lines should first be crossed to flies carrying the Drac1^{N17} or Dcdc42^{N17} transgenes. The resulting flies of the F1 generation should then be crossed to an embryonic Gal4 driver line to produce embryos in the F2 generation that carry the EP element, the Drac1^{N17}/Dcdc42^{N17} and the Gal4 transgenes. Such a procedure is not suitable, however, for several reasons:

First, the EP insertion sites are not known and presumably 50% of the EP elements would segregate from Drac1^{N17}/Dcdc42^{N17} transgenes in the second cross. These EP elements insertions could therefore not be investigated for interaction with Gal4-mediated Drac1^{N17}/Dcdc42^{N17} overexpression phenotypes.

Second, DC is a subtly balanced process that implies a summation of various, also antagonizing forces (see chapter 1.1.) (Kiehart et al., 2000). For example, mutations in both the *Drosophila* JNK, Basket, and its negative regulator, the phosphatase Puckered, lead to similar defects in dorsal closure (Martin-Blanco et al., 1998; Riesgo-Escovar et al., 1996). Furthermore, overexpression of Puckered also leads to a dorsal open phenotype (Martin-Blanco et al., 1998;

Rintelen et al., 2003). It might even be possible that a specific Rac or Cdc42 interactor could both suppress and enhance the dorsal closure phenotype depending on the level of expression. This is supported by the seemingly paradox fact that expression of either dominant negative or constitutive active Dcdc42 can partially rescue the dorsal open phenotype of $Drac1^{N17}$ expressing embryos (Harden et al., 1999).

Therefore, and third, the analysis of these subtle changes of the embryonic phenotype is more time consuming than the analysis of adult phenotypes and hence limits the number of embryos that can be screened. A partial rescue to larval stages or a complete rescue to adulthood will probably occur very rarely.

To overcome these difficulties we took advantage of two tester lines in which eye-specific Gal4 drivers (sev-Gal4 and GMR-Gal4) activate expression of dominant negative versions of either Drac1 ($Drac1^{N17}$) or Dcdc42 ($Dcdc42^{N17}$) by using the Gal4/UAS system (Brand and Perrimon, 1993). In the following paragraphs we will refer to the tester lines as GMR>$Drac1^{N17}$ and sev>$Dcdc42^{N17}$, respectively. The expression of these dominant negative proteins during eye development leads to a disruption of the normal eye structure (Fig. 5 D and F).

The resulting rough eye phenotype, rather than the DC phenotype, was used as a starting point for our gain-of-function screen, assuming that Drac1 and Dcdc42 signaling is conserved in every cell type. Such assumptions are commonly made: Raymond et al., for example, recently showed that overexpressed RacGAP(84C) has the same *in vivo* substrate specificity during eye development and embryonic dorsal closure (Raymond et al., 2001). It has been shown earlier that $Drac1^{N17}$ and $Dcdc42^{N17}$ expression interferes with normal eye patterning (Fanto et al., 2000; Raymond et al., 2004). Fanto et al. reported that the resulting defects of $Drac1^{N17}$ expression were similar to the loss-of-function phenotypes typical of tissue-polarity genes. Furthermore, Genova et al. showed that Dcdc42 mutant clones in the eye fail to produce adult ommatidia (Genova et al., 2000).

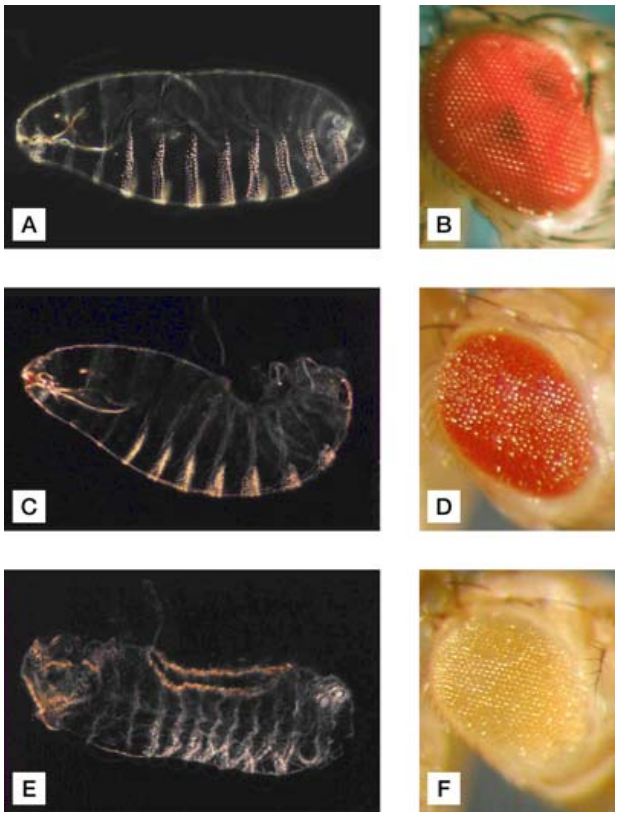


Figure 5. Expression of dominant negative Drac1 and Dcdc42 during embryonic and eye development. (A) Cuticle preperation of a wildtype embryo. (B) Wildtype eye. Overexpression of DN Drac1 (C) and Dcdc42 (E) in the embryo leads to complete failure of dorsal closure. Expression of $Drac1^{N17}$ and $Dcdc42^{N17}$ in the eye using GMR-Gal4 or sev-Gal4, respectively, causes a rough appearance of the eyes due to disordered and partially fused ommatidia (D and F). The causes for this phenotype were not examined in detail but may involve aberrant cytoskeletal behaviours. As in DC, Rho, Rac and JNK signaling have been implicated in planar polarity in the eye (Fanto et al., 2000), thus the disorder of ommatidia could be due to defective planar polarity. This phenotype is dominantly suppressed by the herein identified suppressor EP elements that direct overexpression of a nearby gene (see Fig. 7 in chapter 2.4.).

2.3. Screening for suppressors of a rough eye phenotype

In total we crossed 4900 and 5500 independent EP insertion lines with the GMR>$DRac1^{N17}$ and sev>$Dcdc42^{N17}$ tester lines, respectively (Fig. 6, Tab. 2). A fraction of the EP lines were crossed with both tester lines.

The F1 generation was then screened for modifiers of the rough eye phenotype. Modifiers either improved or worsened eye structure and were referred to as suppressors or enhancers, respectively. Modification of the rough eye phenotype is supposed to be caused by EP

dependent transcription of a nearby gene. These genes thus represent potential Drac or Dcdc42 signaling components.

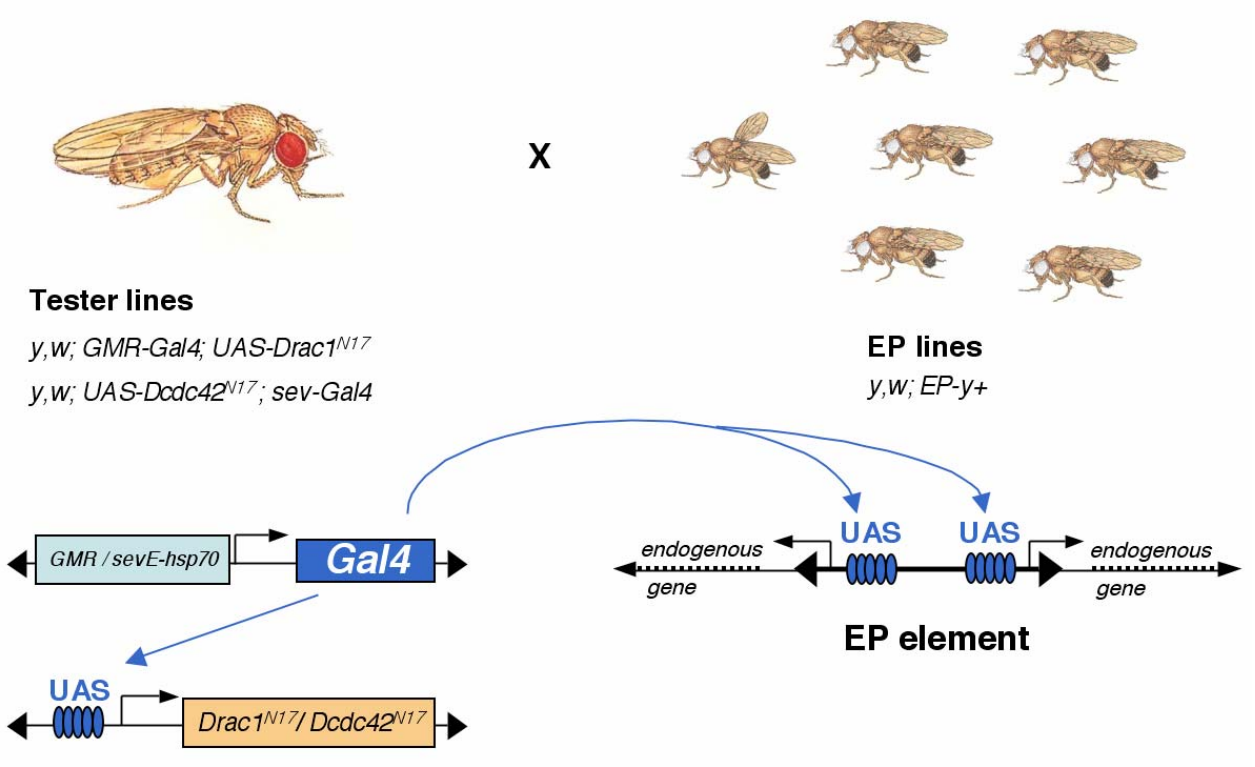


Figure 6. Scheme of the EP suppressor screen

Tester flies carrying the yeast Gal4 transgenes that are under the control of the sev- or GMR-enhancer, activate dominant negative UAS-Dcdc42^{N17} or UAS-Drac1^{N17} specifically in the eye. They have been crossed to a large number of flies with new EP element insertions. The combination of the eye-specific Gal4 with UAS-Dcdc42^{N17} or UAS-Drac1^{N17} gives rise to the rough eye phenotype. The overexpression of a gene X under the control of the UAS/Gal4 system may suppress or enhance the rough eye phenotype, especially when gene X is involved in the same pathway.

2.4. Selection of 22 suppressors specific for either Drac1^{N17} or Dcdc42^{N17}

From the set of 4900 EP lines crossed with GMR>Drac1^{N17} 83 (1.7%) lines behaved as suppressors and 1715 (35%) lines as enhancers of the rough eye phenotype. 173 (3.1%) out of 5500 EP lines suppressed the rough eye phenotype of sev>Dcdc42^{N17} whereas 1069 (19%) were found to be enhancers (Tab. 2).

The high percentage of enhancers suggested that the affected genes are not Drac1 or Dcdc42 interactors but rather cause a disturbance of the eye structure by unrelated mechanisms.

Moreover, the high number of enhancer lines complicates the handling. Taking these considerations into account we decided not to characterize on these lines.

Suppression, that manifests as improvement of the disrupted eye structure, can not be caused by additive effects. Suppression is rather caused by a specific restoration of the disturbed signaling pathways. The view that suppression is more specific is further supported by the low proportion (1.7 - 3.1%) of suppressors found for both tester lines. Thus we continued to analyze the suppressors. We retested the suppressors with the same tester lines as used in the first round. Upon retesting the percentage of confirmed suppressors was lower. This finding can be explained, first, by variability and unspecificity of the phenotypes of the suppressor lines and, second, by application of more stringent selection criteria. After this selection step, 28 suppressors of GMR>Drac1^{N17} and 44 suppressors of sev>Dcdc42^{N17} remained (Tab. 2).

tester line	**y,w; GMR-GAL4; UAS-Drac1^{N17}**	**y,w; UAS-Dcdc42^{N17}; sev-GAL4**
# EP lines	4900	5500
# enhancers (+lethals)	1715 (35%)	1069 (19%)
# suppressors	83 (1.7%)	173 (3.1%)
# suppressors after retest	28 (0.6%)	44 (0.8%)
# specific/exclusive no additional interaction with other tester lines (Table 3)	11 (0.2%)	12 (0.2%)

Table 2. Numbers of screened EP lines and percentages of identified enhancers and suppressors

4900 and 5500 EP lines were crossed with GMR>Drac1^{N17} and sev>Dcdc42^{N17}, respectively. A high percentage of EP lines enhanced the rough eye phenotype of the two tester lines, indicating unspecific effects. These enhancer lines were thus discarded. After retests of the rough eye suppressors 23 lines remained that exclusively interacted with one of the two tester lines.

Next, we entered our results into a custom built database. We then compared the candidate lines with data obtained by other collaborators that participated in the screen with different tester lines. These other collaborators intended to find genes that potentially act in the insulin, TOR, myc, Wg or Hh signaling network (Tab. 3). The lines that did not exclusively suppress the GMR>Drac1^{N17} or sev>Dcdc42^{N17} phenotypes were considered to be less specific and were thus discarded. However, genes that participate in several pathways might be missed with this approach. For example, it is conceivable that components of the Wg signaling pathway modify

the phenotype of both tester lines sev-Wg and GMR>Drac1^{N17}, since it has been shown that wingless signaling components and Drac1 work in a common pathway to affect planar polarity in the eye (Fanto et al., 2000; McEwen et al., 2000).

After this selection step 23 suppressors fulfilled our strict criteria. Surprisingly, of these 23 suppressors only one (EP43-018, specified as one of 11 GMR>Drac1^{N17} suppressors, not included in the 13 sev>Dcdc42^{N17} suppressors, see Tab.4) was shared by both tester lines, although it is conceivable that many signaling components are common to both RhoGTPases.

The database also shows that an EP dependent enhancement phenotype is found many times in several apparently unrelated tester lines (data not shown). This is typical for unspecific interactions and further supports our previous suggestion that analysis of enhancers is inappropriate for our purpose .

overexpressed gene	**eye- or wing-specific Gal4 drivers**
UAS-Dcdc42^{N17}	*sev-Gal4*
UAS-Drac1^{N17}	*GMR-Gal4*
UAS-InR	*GMR-Gal4*
UAS-PKB, UAS-PDK1	*GMR-Gal4*
UAS-Baboon^{QD}	*GMR-Gal4*
UAS-myc(3)	*GMR-gal4*
UAS-S6K	*ap-Gal4*
sevE-hsp-sev^{S11}	*sEsP-Gal4*
** sev-wg*	*GMR-Gal4*
UAS-lgs17E	*sal-Gal4*
UAS-ptc	*sal-Gal4*

UAS-smo [l]	*sal-Gal4*

* Gal4 independent wg-expression

Table 3. Various additional tester lines of EP screen collaborators

Several collaborators screened the EP lines with different tester lines that display either specific eye or wing phenotypes (not shown). Eye-specific overexpression of either sev^{S11} (activated form of the *sevenless* receptor tyrosine kinase), $Dcdc42^{N17}$, $Drac1^{N17}$, $Baboon^{QD}$ (activated form of *Drosophila* Activin receptor) or *wg* lead to a disruption of the normal eye structure. *GMR-Gal4* dependent overexpression of either *InR,* combined *PKB* and *PDK1,* or *dMyc* (three copies) leads to overgrowth of the eyes. Overexpression of *dS6K* in the dorsal part of the developing wings using *ap-Gal4* leads to overgrowth of this area and subsequently to a bending down of the wings. *sal-Gal4* dependent overexpression of either *lgs17E*, *ptc* or *smo[l]* lead to patterning defects of the adult wing.

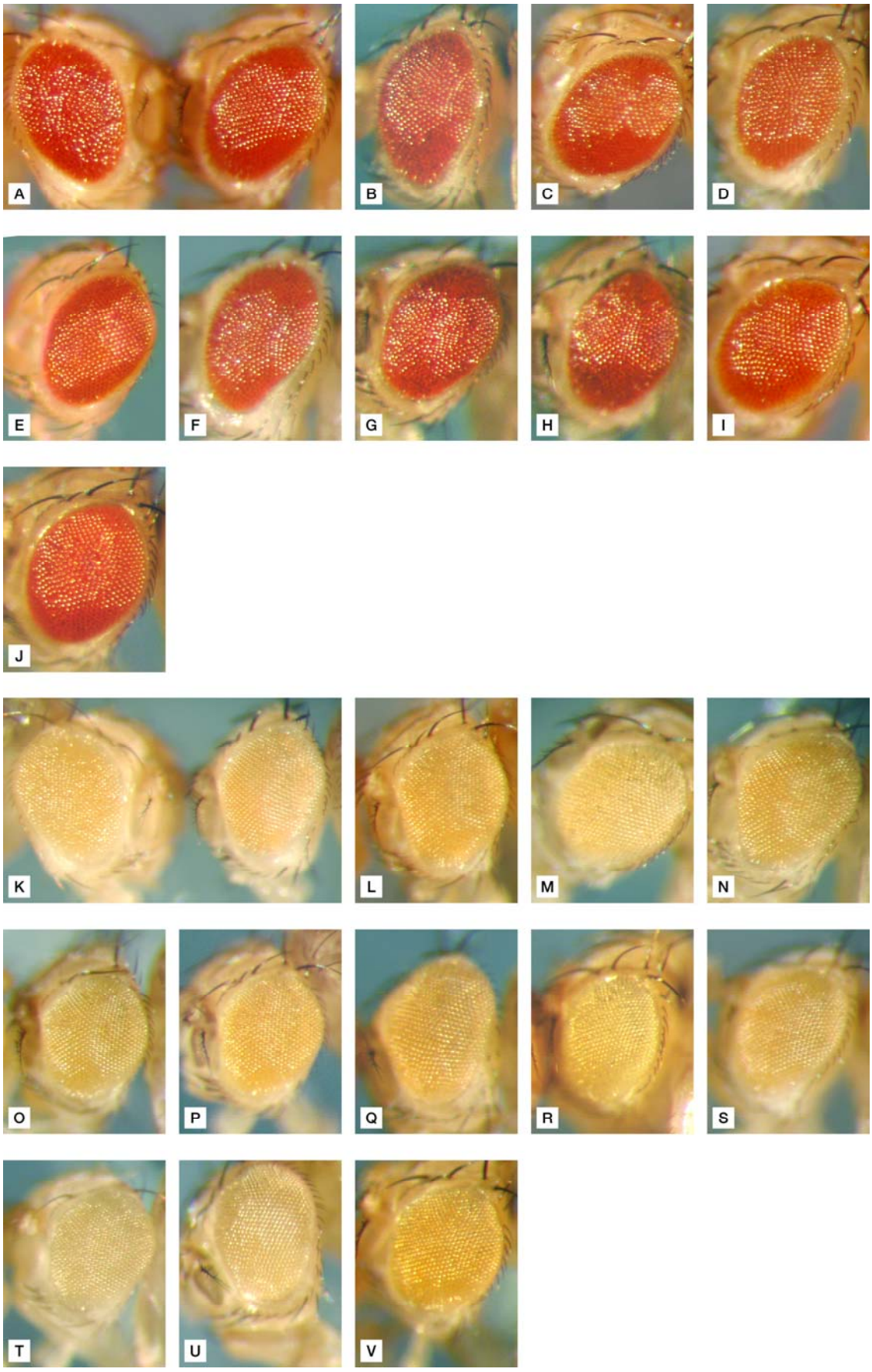

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Figure 7. Overview of the rough eye suppressor phenotypes.

On the left of (A) the rough eye phenotype of the GMR>DRac1^{N17} tester line is shown (see also Fig. 5D). A rough eye is characterized by an irregular array and disrupted structure of ommatidia. Improvement of eye structure (i.e. suppression of the GMR>DRac1^{N17} rough eye phenotype) is seen when the selected EP suppressors are co-overexpressed, (A) right side and (B-J). (A, right side) EP32-120 (responsible gene: *MSP-300*), (B) EP51-198 (*MSP-300*), (C) EP36-054 (*SelD*), (D) EP43-018 (CG30122), (E) EP34-026 (*SA-2*), (F) EP39-161 (*falafel*), (G) EP47-082 (*falafel*), (H) EP55-127 (*cpo*), (I) EP58-173 (*cpo*), (J) EP53-065 (*HLHm7*).

Eye-directed overexpression of Dcdc42^{N17} also leads to a rough eye phenotype (K) left side (see also Fig. 5F), which is suppressed by co-overexpression of suppressors specific to the sev>Dcdc42^{N17} tester line (K) right side and (L-V). (K, right side) EP31-149 (*Rbf*), (L) EP13-034 (*Trf2*), (M) EP29-104 (*ATP7*), (N) EP29-062 (*e(y)3*), (O) EP43-011 (CG6700), (P) EP33-095 (CG30188), (Q) EP14-215 (CG30421), (R) EP22-048 (CG30421), (S) EP33-059 (CG30421), (T) EP33-094 (RE29262 cDNA), (U) EP22-081 (*Aats-Glupro*), (V) EP-28-221 (*stg)*.

2.5. Tissue-specific overexpression of putative genes affected by EP element insertion

The rough eye modification of the suppressors already suggested a function of the corresponding proteins in Rho-GTPase signaling. We aimed at further investigating the function of the genes during development. To this end, we overexpressed the selected EP suppressors in different tissues and analyzed the resulting phenotypes.

We crossed the EP lines to different Gal4 lines that drive expression either in the embryo, in the imaginal discs, during pupal thorax closure or ubiquitously during the entire development (Tab. 4 and Fig. 8):

The enhancer trap line 69B drives expression of UAS containing sequences in the embryonic ectoderm from stage 11 and in the eye-antennal, haltere, leg and wing imaginal discs. MD237 is a Gal4-containing P-element enhancer trap line in the pnr locus which is expressed in the dorsal-most regions of the embryo and the imaginal discs (Calleja et al., 2000). Gal4 fused to glass multiple reporter (GMR-Gal4) is expressed in post-mitotic cells of the developing eye, and actin(5C)Gal4 drives ubiquitous expression throughout development.

We found that 10 lines produced a visible phenotype when driven by one of these Gal4 lines. 6 out of 13 tested EP lines caused embryonic or pupal lethality in combination with Actin(5C)Gal4. When using 69B as source of Gal4, two EP elements produced penetrant dorsal closure and head involution defects (EP34-054, EP36-026, Fig. 8 A, B, respectively). Embryos with 69B and

EP28-221 displayed a defective germ band retraction. Two lines (EP39-161 and EP47-082) were semi-viable in combination with 69B and displayed a specific wing phenotype (Fig. 8 G). Furthermore, some dead embryos with dorsal open phenotypes were observed (Fig. 8 C).

In four lines MD237 driven overexpression produced a cleft in the thorax of adult flies (two examples are shown, EP31-149 (*Rbf*) and EP47-082 (*falafel*), Fig. 8 E and F, respectively). This defect results from a failure in the fusion of the two imaginal wing discs during metamorphosis. The closure of the thorax during metamorphosis resembles the process of dorsal closure during late embryogenesis. In addition to morphological similarities, genetic evidence points to similar molecular mechanisms that direct embryonic dorsal and imaginal thorax closure, involving the JNK and Dpp signaling pathways (Agnes et al., 1999; Martin-Blanco et al., 2000; Zeitlinger and Bohmann, 1999).

One EP line, EP53-065, led to missing bristles on the thorax and abdomen upon MD237 driven overexpression. Loss of bristles was also observed when this EP line was expressed in the eye. Nevertheless, the suppression of the rough eye phenotype was obtained by a clear improvement of the array and individual structure of ommatidia. The gene responsible for the suppression and overexpression phenotype is HLHm7 (Tab. 4, Chap. 2.6. and 2.9.6.), which is known to play a role in neural development and the formation of mechanosensory bristles.

Another line, EP29-104, displayed a pale cuticle of adult flies upon both MD237 and actin(5C)Gal4 driven overexpression (Tab. 4 and Fig. 8 H). In this case *ATP7*, a gene encoding a copper transporter, is responsible for the mutant phenotype. Consistently, misregulation of copper homeostasis has been shown to affect pigmentation in *Drosophila* (Zhou et al., 2003). This effect could be due to the requirement for copper of tyrosinase, a copper-dependent enzyme involved in melanogenesis and pigmentation. Petris et al. showed that human tyrosinase was inactive in ATP7A mutant fibroblast cells (Petris et al., 2000). The role of *ATP7* in pigmentation is further described in *Drosophila* as well (Norgate et al., 2005).

Thus, although the overexpression phenotypes do not necessarily define the normal function of these genes during development, our results indicate that meaningful dominant effects can be uncovered. Furthermore, overexpression of some of the EP lines interferes with developmental processes that are known to be dependent on functional Rho GTPase signaling, such as dorsal closure (reviewed by Harden 2002) and thorax closure (Ishimaru et al., 2004). The genes causing these phenotypes therefore represent putative new DC genes and Rho GTPase interactors, the identification of which was the initial purpose of the screen.

The results of the overexpression experiments are summarized in Table 4, examples of the overexpression phenotypes are shown in Figure 8.

Figure 8. 69BGal4 and *pnr*Gal4 mediated overexpression phenotypes of EP element lines.
69BGal4 dependent overexpression phenotypes of EP element insertion lines affect embryogenesis. Defects in dorsal closure, head involution and germ band retraction in (A-D). (A) 69B>EP36-054 (responsible gene: SelD), (B) 69B>EP36-026 (SA-2), (C) 69B>EP47-082 (*falafel*), (D) 69B>EP28-221 (*stg*). (G) Wing defects in flies with 69B>EP47-082 (*falafel*). (E, F, H) *pnr*Gal4 dependent overexpression phenotypes of EP element insertion lines affect thorax closure and adult cuticle pigmentation. (E) *pnr*Gal4>EP31-149 (*Rbf*), (F) *pnr*Gal4>EP47-082 (*falafel*), (H) *pnr*Gal4>29-104 (*ATP7*), cuticle on thorax and abdomen is brighter in the *pannier* domain.

2.6. Identification of the genes responsible for the suppression/ overexpression phenotypes

2.6.1 Recovery of DNA sequences flanking the EP element insertions by plasmid rescue

To characterize the genes responsible for the suppression and overexpression phenotypes, the DNA flanking the EP elements was isolated by plasmid rescue and the genomic region immediately downstream of the EP element promoter was sequenced.
The obtained sequence was subjected to Blast searches against the fully sequenced genome of *Drosophila* using http://flybase.net/blast/. We then searched the region 5' and 3' of the insertion site for known or predicted genes. We found that 21 out of the 23 EP elements were inserted 5' of the translational start site of at least one predicted or cloned gene suggesting that activation of gene transcription caused the suppression of the $Drac1^{N17}$/$Dcdc42^{N17}$ rough eye phenotype. The remaining two genes were located as follows: EP33-094 was inserted in a genomic region where

no obvious genes are located in the close vicinity. However, an expressed sequence tag (EST RE29262) of a cDNA aligns near the EP element insertion site. This locus was also identified in another misexpression screen which was designed to identify genes that, upon overexpression, cause defects in the male germ cells (Schulz et al., 2004). A second EP element (EP51-198) was inserted in the third exon of the gene encoding muscle specific protein 300 (MSP-300). This gene was hit by a second EP element (EP32-120). However, EP32-120 was located 5' of the gene and is therefore likely to drive the expression of the full length transcript. All the suppressors with corresponding genes are listed in Table 4.

2.6.2. From double- to single-headed EP elements to identify the responsible genes

To distinguish whether the gene 5' or 3' of the EP element is responsible for the suppressive effect we crossed CRE recombinase expressing flies with those carrying the EP-elements. This led to the excision of the UAS sequences at the 5' end of the EP element (see also Fig. 4). The resulting single headed EP element lines were then tested for suppression of the rough eye phenotype and their additional possible overexpression phenotypes. If the phenotype was lost after excision of the 5' UAS sequences, we concluded that the responsible gene was located 5' of the EP element. On the other hand, if the phenotype remained unchanged the gene was located 3' of the EP element. However, the lines EP13-034 and 14-215 carried EP elements with defective CRT sites which prevented excision of the 5' UAS sites. Another complication arose in 4 lines, containing presumably functional CRT sites. No flies were obtained where the y^+ marker gene was excised by the cre recombinase. In summary, for 16 of the 23 EP lines, we were able to identify single genes that are probably responsible for the suppression phenotypes and hence are potential DRac1 or Dcdc42 interactors (Tab. 4).

In those lines where CRE-mediated excision of UAS sequences was not possible, we continued as follows: In the case of EP13-034, we made use of a previously published EP element EP(X)1310 (obtained from the Bloomington stock center). This single headed EP element is located at a similar position as EP13-034 but points to CG11190 only. This EP element, however, did not behave as a suppressor of the rough eye phenotype indicating that Trf2 rather than CG11190 is responsible for the suppression phenotype.

In the case of EP22-081, we decided to overexpress one of the two candidate genes. We constructed an UAS-transgene containing the cDNA of AP-1 sigma. Co-overexpression of this transgene with sev>Dcdc42^{N17}, however, did not result in suppression of the rough eye phenotype. This suggests that Aats-glupro rather than AP-1 sigma is responsible for

suppression of the rough eye phenotype. Since these two genes are positioned very close to each other (327bp) and the EP element inserted only 9bp upstream of the transcriptional start site of Aats-glupro we evaluated the possiblity that the EP element itself leads to a loss-of-function situation. For that reason, we tested a deficiency strain in which the two genes were deleted and examined whether it also suppressed the rough eye phenotype of sev>Dcdc42^{N17}. This was not the case.

EP14-215 was inserted at exactly the same position as both EP22-048 and EP33-059. Cre/lox experiments for the latter two EP elements identified CG30421 as the gene responsible for the suppression phenotype. We therefore concluded that EP14-215 also drives CG30421 (see also Chap. 2.9.2.).

The DNA sequence adjacent to EP33-095 aligns with the sequence of an expressed seqence tag (SD24970) which contains no open reading frame. Furthermore, there are no other ESTs in the same region. We therefore assume that SD24970 does not represent a real gene but an artefact. CG3162 is located 3' of the EP element, at a distance of 6kb. However, the orientation of the gene would lead to an antisense transcript if driven by the EP element. The best candidate gene is situated 5' of the EP element. The transcription start site of the cDNA RE72153 is 502 bp away from the EP element insertion. This cDNA is partially identical with the gene annotated as CG30188, thus RE72153 possibly represents an alternatively spliced transcript.

EP55-127 and EP58-173 were inserted at exactly the same position in the *couch potato* (*cpo*) locus. The insertion site was 22kb upstream of the translation start site and 12kb downstream of the transcription start site, the third (untranslated) exon however starts only 29 bp downstream (3') of the EP element. Rim, encoding a Rab3A effector, is the next gene located 5' of the EP element, however at a huge distance of 35 kb. We therefore assume that *cpo* is the gene responsible for the suppression phenotype.

In summary, after the plasmid rescue and CRE/lox experiments, we could determine single genes probably responsible for the suppression phenotype in 17 of the 23 EP lines. No gene except for an EST could be assigned to one EP line (EP33-094). In the case of the five remaining EP lines one of the respective two candidate genes 5' and 3' of the EP element could be ruled out by several means: a) by testing a publically available single-headed EP element (CG11190), b) by testing a UAS construct (AP-1 sigma) and c) by sequence analysis. In consequence we had 23 EP suppressor lines corresponding to 17 genes (Tab. 4).

	gene responsible for supression or candidate genes	cytological position	EP line	insertion site relative to ATG	overexpression phenotypes: 69B-Gal4	pnr-Gal4	actin-Gal4
Dcdc42^{N17} suppressors	Rbf	1C5	31-149	-565 bp	lethal	thorax cleft (Fig. 8 E)	ND
	Trf2, (CG11190)	7E7-9	13-034	-12602 bp (ATG of Trf2)	WT	WT	lethal
	ATP7	10F1-2	29-104	-588 bp	WT	pigmentation defect in pnr domain (Fig. 8 H)	pale cuticle, less bristles
	e(y)3	18D8-11	29-062	-742 bp	WT	WT	ND
	CG6700	32B1	43-011	-376 bp	WT	WT	WT
	CG30188, (SD24970)	59D1	33-095	-13234 bp (ATG of CG30188)	WT	WT	ND
	CG30421	60D16-E1	14-215 22-048 33-059	-7497 bp	WT WT WT	WT WT WT	WT WT WT
	RE29262	72D9	33-094	-34 bp (transcription start site)	WT	WT	ND
	Aats-Glupro, (Ap-1 sigma)	95D1	22-081	-91 bp (ATG of AatsGlupro)	WT	variable slight thorax cleft	WT
	stg	99A5	28-221	-470 bp	germ band retraction defects (Fig. 8 D)	slight thorax cleft, less bristles	ND
Drac1^{N17} suppressors	pfrx	18C8	31-137	-467 bp	ND	ND	ND
	MSP-300	25C7-10	32-120	+38644 bp (see text)	WT	WT	lethal
			51-198	-657 bp	WT	WT	WT
	SelD	50E8	36-054	-246 bp	anterior open (Fig. 8 A)	WT	lethal
	CG30122 (also weak suppressor of Dcdc42^{N17})	55E3	43-018	-243 bp	WT	WT	WT
	SA-2	62A1	34-026	-8001 bp	anterior open (Fig. 8 B)	ND	ND
	CG9351 *(falafel)* (Chap. 3)	87F10-11	39-161	-3891 bp	semilethal – dorsal closure defects (Fig. 8 C, Fig. 11 F)), wings bent downwards (Fig. 8 G, Fig. 11 E)	thorax cleft (Fig. 8 F, Fig. 11 B)	pupal lethal
			47-082	-3901 bp			
	cpo, (Rim)	90D1-E1	55-127	-22486 bp (ATG of cpo)	ND	ND	ND
			58-173		ND	ND	lethal
	HLHm7	96F10	53-065	-247 bp	WT	less bristles in pnr domain	less bristles

Table 4. Identified genes and phenotypic effects of induced expression in different tissues
List of all 23 EP insertions that were selected because they suppressed the rough eye phenotype of flies expressing either Dcdc42N17 or Drac1N17.
To identify the loci of the EP insertions, the flanking DNA was isolated by plasmid rescue and the genomic region immediately downstream of the EP element promoter was sequenced. Since the EP-element used in this study has UAS sequences at both ends, CRT-sequences flanking the UAS sequences at the 5' end were excised by CRE-recombinase. The flies with these "halved" EP elements were then retested for suppression. The genes that were found to be responsible for the suppression are indicated. In the cases where the CRE/lox experiment did not yield a result, one of the respective two candidate genes 5' and 3' of the EP element, respectively, was ruled out (genes in brackets) by other means (see text).
The distance between the EP element insertion site and the start of the open reading frame (as predicted by the Berkeley *Drosophila* Genome Project) is indicated.
Overexpression: Gene expression driven by EP insertions was activated using the Gal4 driver lines 69B, pnr-Gal4 and actin-Gal4. 69B is expressed in the embryonic ectoderm from stage 11 and in the eye-

antennal, haltere, leg and wing imaginal discs. pnr-Gal4 is expressed in the dorsal-most regions of the embryo and the imaginal discs. actin-Gal4 is ubiquitously expressed throughout development.

2.7. Selection of two lines for further investigation

The remaining 23 EP lines are specific suppressors for either GMR>DRac1^{N17} and sev>Dcdc42^{N17} and hence potentially play a role in RhoGTPase signaling.
In the next step I evaluated and discussed each of the 17 genes corresponding to the 23 EP element insertions (see discussion). Based on these considerations I selected two genes that seemed particularly promising for further investigation: CG30421 and CG9351 (*falafel*).

2.7.1. Overexpression of CG30421 leads to suppression phenotype

The three EP element lines EP14-215, EP22-048 and EP33-059 were identified as suppressors of the sev>Dcdc42^{N17} rough eye phenotype. All three EP elements are inserted at exactly the same position on the right arm of the second chromosome at the cytological position 60D16-60E1 (Fig. 9). EP14-215 had nonfunctional CRT sites, hence CG18510 which lies 5' to the EP element could not be excluded to be responsible for suppression. The two other EP lines, however, had functional CRT sites. The orientation of the EP element with respect to the CRT-flanked UAS seqences is different in these two lines. After excision of the UAS sequences the expression of CG30421 was maintained in EP22-048 (hereafter referred to as EP22-lox) but abolished in EP33-059. Only EP22-lox still suppressed the rough eye phenotype indicating that CG30421 was the responsible gene. To confirm this we cloned the open reading frame of CG30421 into the pUAST vector (Brand and Perrimon, 1993). As expected we found that co-overexpression of UAS-CG30421 and sev>Dcdc42^{N17} suppressed the rough eye phenotype.

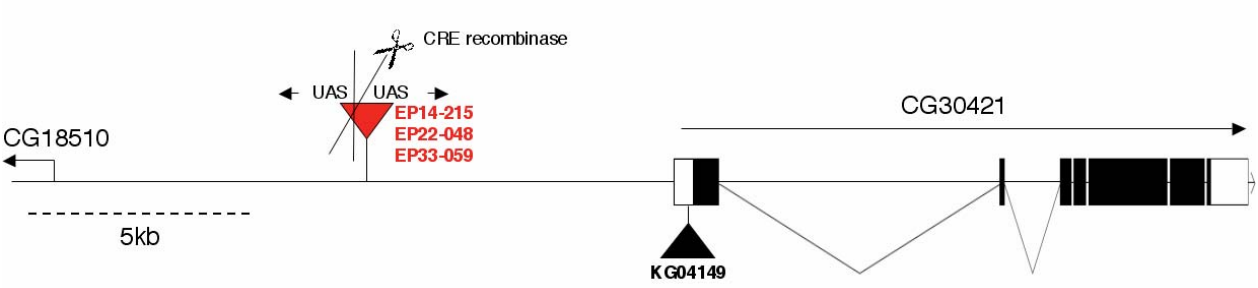


Figure 9. Gene locus of CG30421 and P-element insertion sites

The red triangle marks the insertion site of the 3 EP elements 14-215, 22-048 and 33-059, which is at a 7kb distance from the transcription start site (left white box). The black boxes designate the ORF. Another insertion of a new P-element, KG04149, that could be used for a jump-out screen (see below), is represented by the black triangle.

In the first release of the *Drosophila* genomic sequences, CG30421 was incorrectly annotated as two separate genes, CG9189 and CG3872. This was due to the finding that several cDNAs with expressed sequence tags (ESTs) spanned only parts of CG30421. I found that one cDNA, however, had a 5' EST (GH27809.5prime) belonging to CG9189 and a 3' EST (GH27809.3prime) belonging to CG3872. A later annotated full-length sequence of this cDNA confirmed that these two genes are in fact only one.
CG30421 codes for a putative ubiquitin specific protease. Such enzymes cleave off ubiquitin residues of specific substrates and thereby prevent polyubiquitination and subsequent degradation of these substrates by the proteasome complex. Since CG30421 overexpression suppresses dominant negative Dcdc42, targets of CG30421 are likely to be positive regulators Dcdc42. A particularly interesting target would be Jun, which is known to be regulated by polyubiquitination in human cells (Wang et al., 2001) and has been shown to act downstream of Dcdc42 during the process of thorax closure in *Drosophila* (Agnes et al., 1999).

2.7.2. Reversion of overexpression phenotypes of *CG30421* by EMS mutagenesis

CG30421 overexpression did not lead to any visible phenotypes when driven by either pnr-GAL4 (MD237), actin(5C)Gal4 or 69B (see above). So far the only functional information of CG30421 is the suppression of the rough eye phenotype in the sev>Dcdc42^{N17} tester line.
In order to further address the function of CG30421 it is important to know the consequences of loss-of-function mutations. For this purpose I performed a mutagenesis screen by making use of the suppression phenotype caused by the EP-dependent overexpression of CG30421.
Males containing EP22-lox were treated with ethyl methanesulfonate (EMS) and were subsequently crossed to females of the sev>Dcdc42^{N17} tester line. The F1 generation was then screened for flies that showed a reversion of the suppression phenotype, i.e., flies in which EP22-lox was no longer suppressing the Dcdc42^{N17} rough eye phenotype. These flies potentially carry mutations in CG30421. Unexpectedly I found a high proportion of revertants (10-20%).
As I used a standard dosis of EMS, which is expected to induce one mutation every 100kb, the probability that specific mutations in CG30421 are responsible for all reversion events is close to zero. What could be the cause for the high proportion of revertants? Since EP22-lox does not

lead to a complete suppression of eye roughness back to wild type, this suppression phenotype may be too sensitive for second site modifiers. Such mutations do not necessarily affect genes involved in Dcdc42 signaling but may rather unspecifically interfere with eye development. Thus, reversion of suppression could be compared to the enhancement of the sev>Dcdc42^{N17} rough eye phenotype that was obtained in a similarly high proportion of EP lines (Tab. 2).

Mutagenesis screens for reversion of an EP-dependent phenotype have been previously performed in our lab. For example, a very similar mutagenesis screen for revertants of a suppressor of the *Baboon*^{QD} (Tab. 2) rough eye phenotype also failed because of improbably high numbers of revertants (S. Breuer, personal communication). However, a screen for reversion of suppression of an overgrowth phenotype *(GMR>InR*, Tab. 2*)* was performed successfully (Wittwer et al., 2005). The difference between the two screens may be that in one case the reversion event is overgrowth, which probably occurs less frequently than an enhancement of disruption of eye structure, i.e., reversion of the suppression phenotype.

A characteristic and robust overexpression phenotype would be better suited for a reversion mutagenesis. In this case, the reversion would manifest as an improvement of a defective phenotype, which is in contrast to the reversion of a suppression phenotype, which manifests as an enhancement of a subtle phenotype. However, EP-dependent overexpression of CG30421 does not lead to a visible phenotype (Tab. 4) that could be used for a mutagenesis screen. For example, EP-dependent overexpression of dMKP-3 in the eye led to a rough eye phenotype, and a mutagenesis screen for reversion to wild type eyes has been performed successfully (Rintelen et al., 2003).

Thus, the function of CG30421 remains to be assessed. In summary, the gene was identified in an overexpression screen as a potential regulator of Dcdc42. However, overexpression phenotypes may result from unspecific effects rather than reflecting the physiological function of the gene. A better understanding of the precise function of CG30421 can be expected from the analysis of a loss-of-function mutation. However, a reversion mutagenesis screen to obtain mutants of CG30421 failed. Another possibility to generate mutations in CG30421 is a jump-out screen. This method requires the imprecise excision of a P-element that is inserted near the gene of interest. In the case of CG30421, the EP elements identified in our screen are located too far away (7kb) from the translational start site of the gene. A publically available P-element, KG04149 (Fig. 9), is located only 175 bp away from the ATG and could serve as a tool for doing a jump-out screen.

2.7.3. CG9351/*falafel*

Mutagenesis of CG9351 was successfully perfomed. The results are presented in chapter 3.

2.8. General discussion of the EP screen

The aim of this study was to identify novel signaling components that contribute to dorsal closure of the *Drosophila* embryo. For this purpose I performed a gain-of-function screen for genes that interact with either Drac1 or Dcdc42, which are known key players in DC. A large number of flies with random and independent EP element insertions were crossed to flies that express dominant negative forms of either Drac1 or Dcdc42 specifically in the eye. The consequent rough eye phenotype of the Drac1/Dcdc42 tester flies was suppressed by a number of EP element insertions. After further strict selection steps 23 EP lines remained that exclusively suppressed the Drac1 or Dcdc42 phenotypes. These suppressor lines are therefore potential interactors of Drac1 or Dcdc42. The recovery of the DNA sequences near the EP element insertions allowed us to find the genes that are responsible for the suppression phenotype. This approach led to the identification of 17 genes, none of which has been previously reported to interact with Drac1 or Dcdc42. The potential role of these genes in DC has not yet been investigated except for one gene, *falafel* (Chap. 3).
In the following I discuss the characteristics of our approach and both its advantages and limitations. After this, each of the identified genes will be discussed (Chap. 2.9.) and evaluated for further use in investigation.

2.8.1. Advantages and limitations of our screening strategy

Genes involved in dorsal closure have been identified in conventional loss-of-function screens. However, this approach has limitations because mutations in a high proportion of *Drosophila* genes do cause pleiotropic phenotypes or do not result in phenotypic abnormalities (Ashburner et al., 1999; Miklos and Rubin, 1996).
To complement the loss-of-function screens and to find novel genes involved in dorsal closure we decided to perform a gain-of-function screen.

We misexpressed dominant negative versions of the two RhoGTPases Drac1 and Dcdc42 in the eye and used the resulting rough eye phenotype as a starting point for our genetic screen. The phenotype caused by overexpression results from the increased presence and function of a protein. The non-physiological high level of a protein, however, may perturb development also unspecifically: It may lead to non-specific protein-protein interaction, to non-physiological

distribution and formation of aggregates. All this may disturb the normal function and viability of a cell and thereby produce a non-specific phenotype. However, Fanto et al. observed that overexpression of a dominant negative form of Rac1 resulted in defects that were similar to the loss-of-function phenotypes typical of Drac1 interacting genes (Fanto et al., 2000). This finding makes us confident that the rough eye phenotype caused by $Drac1^{N17}$/$Dcdc42^{N17}$-overexpression indeed reflects a specific phenotype of defective RhoGTPase signaling.
Another general concern with respect to overexpression by the UAS/Gal4 system is that Gal4 on its own can cause developmental defects and apoptosis in the eye (Kramer and Staveley, 2003). This suggests that apoptosis inhibitors would also suppress Gal4 mediated phenotypes. I discarded EP lines that were found to suppress also other Gal4-dependent phenotypes of other tester lines suggesting that my selection does not include apoptosis inhibitors.

Another concern is that Rac or Cdc42 signaling may not be common to all cell types. Based on the assumption that these signaling pathways are common to all cell types we performed the EP screen for eye rather than DC phenotype modifiers. This approach was useful because the viability of the corresponding tester lines allowed an F1 screen. Although this assumption is widely accepted in developmental biology, any conclusion must be interpreted with caution. The function of particular proteins in a signaling pathway may vary depending on cell type and circumstances, and thus it is not always straightforward to extrapolate from one system to another. Thus it is possible that eye specific components of Drac1/Dcdc42 signaling might have been selected in the EP screen that have no function in DC. However, it was shown for a regulator of Drac1, RacGAP(84C), that it has the same *in vivo* substrate specificity during eye development and embryonic dorsal closure (Raymond et al., 2001), suggesting that the use of an eye phenotype is appropriate for screening for DC genes.

2.8.2. Expectations and findings of screening strategy

Our screening strategy together with the above mentioned limitations allows to formulate a prediction of what can be identified:

2.8.2.1. Suppressors, not enhancers

I observed both suppression and enhancement of the rough eye phenotype mediated by overexpression of DN Drac1 and Dcdc42. Enhancement was produced by a large number of EP lines. However, many genes that are overexpressed in the eye lead themselves to a rough eye

phenotype and therefore are thought to enhance a rough eye phenotype in an additive rather than in a synergistic manner. For this reason we discarded the enhancers.
However, when seeking the EP screen database I found plasmid rescue sequence data of other screen participants for a number of enhancers of $Drac1^{N17}$ or $Dcdc42^{N17}$. One of these enhancers turned out to be *aop/yan* which is a known negative regulator of both Ras/MAPK and Drac1/JNK signaling. Thus, it is possible that other "true" regulators of Drac1 and Dcdc42 signaling have been missed by discarding the enhancer EP lines.
Suppressors are much more specific because they improve a disturbed eye structure which is unlikely to be caused by additive effects. Suppression is rather achieved by a specific restoration of Drac1/Dcdc42 signaling. Accordingly we found a low number of suppressors.

2.8.2.2. Positive regulators down- or upstream of Dcdc42/Drac1

Suppressors of the $Drac1^{N17}$/$Dcdc42^{N17}$ phenotype are likely to be positive regulators of Drac1 or Dcdc42 signaling. This includes not only proteins that exclusively interact with Drac1 or Dcdc42 but also those that interact with the GEFs, as discussed above. These regulators can act either upstream or downstream of Rac1 or Cdc42 signaling. In a complete loss-of-function situation of Dcdc42 or Drac1 only proteins downstream are able to restore signaling. But overexpression of $Drac1^{N17}$/$Dcdc42^{N17}$ probably leads to a hypomorphic situation: Endogenous Drac1/Dcdc42 is still active, but probably to a lesser extent because many GEFs are sequestered. Thus upstream regulators can act as suppressors by activating the endogenous proteins.

2.8.2.3. The screen did not identify known components

RhoGTPase signaling has been analyzed extensively and many regulators, such as the GEFs, and downstream effectors, such as members of the JNK pathway, have been shown to play a role in DC, eye development and/or various other processes. Our screen did not identify any of these known components of RhoGTPase signaling.
Several factors may have hindered the isolation of these genes:
1. Our screen did not reach saturation. The 4900 and 5500 EP insertions, that we screened, could have activated the expression of theoretically 25%-40% of all *Drosophila* genes (estimated gene number: 13400-21000 (Adams et al., 2000). However the number of targeted genes is certainly lower in our screen as not all EP elements will be inserted at positions that allow them to activate expression of full length transcripts. Moreover P-elements do not insert randomly in the genome (Spradling et al., 1995) but the genome contains regions where P elements

preferentially integrate ('hot spots'). Also in our study EP insertions driving the same gene were isolated repeatedly (see Tab. 4).

2. Many proteins need to be activated to exert their function, for example by phosphorylation. In such a case, overexpression alone is not sufficient to cause a specific phenotype. This seemed to be the case for the members of the JNK pathway: When I tested UAS-transgenes of the *Drosophila* JNKK, JNK and Jun I observed only a very weak suppression of the GMR>Drac1^{N17} or sev>Dcdc42^{N17} phenotypes (data not shown). Such a weak suppression phenotype would not have been selected in the EP screen.

3. The fact that none of the GEFs have been identified may be explained by their significant redundancy; there are more than 20 members of this protein family apparent from the *Drosophila* genome (Adams et al., 2000). Possibly more than one GEF is required to restore the dominant negative Rac/cdc42 signaling.

Recently a gain of function screen similar to ours was performed (Raymond et al., 2004). Raymond *et al.* also intended to find positive regulators of Rac signaling. They expressed a negative regulator of Drac1, RacGAP84C (instead of Drac1^{N17} in our case), in the eye and screened the flies with the resulting rough eye phenotype for suppressing EP element insertions. Despite the similarity between their and our tester lines very different sets of genes were recovered in the two cases. How can two similar misexpression screens generate such different outcomes?

RacGap(84C) probably targets not only Drac1 but also other small GTPases. This is suggested by Raymond et al. since they found several genes as enhancers or suppressors of RacGAP(84C) that are known to interact with the small GPTases Ras or Rho. We did not characterize the enhancers. Furthermore, we also discarded suppressors that were not exclusively specific for either Drac1^{N17} or Dcdc42^{N17}. Raymond et al. however described several enhancers (that we missed) and suppressors that also interacted with other tester lines. They described only 6 suppressing EP insertions corresponding to 4 genes and 24 enhancers corresponding to 20 genes

The only gene that was identified in both screens is *cpo*. However, *cpo* overexpression leads to suppression of the Drac1^{N17} phenotype, whereas the RacGAP(84C) phenotype is enhanced. Since both Drac1^{N17} and RacGAP(84C) are expected to inhibit Drac1 signaling it is surprising that *cpo* was identified as both suppressor and enhancer. Moreover, the EP(3)3395 and EP(3)3608, which are responsible for this enhancement phenotype of RacGAP, and EP55-196 and EP58-173 are inserted at the very same position 22 kb 5' of the translation start site of the *cpo* gene.

Albeit all this, we certainly analyzed a significant part of the genes of *Drosophila* for interactions with RhoGTPase signaling.

2.9. Discussion of individual EP lines

In the following I discuss each of the identified genes and evaluate their use for further investigation.

For most of the genes the connection to Rho GTPase signaling is not clearly evident. In addition, the identified genes represent a heterogenous group of genes with diverse functions. A classification into groups of genes with common functions was at first sight not compelling. However, after an extensive literature search I hypothesize a common function for six genes in mediating Rac/Cdc42 dependent regulation of homeostasis of reactive oxygen species (ROS). These six genes are Trf2, ATP7A, CG6700, CG30421, pfrx and SelD (found in either Cdc42 or Rac screen). Thus, a short introduction into the mechanisms and effects of ROS generation and their regulation by Rac and Cdc42 (Chap. 2.9.1.) precedes the discussion of the six genes (Chap. 2.9.2.).

Furthermore, I put Rbf and stg into one chapter (Chap. 2.9.4.) based on their function in cell cycle control.

Finally, the apparently unclassifiable genes are discussed (Chap. 2.9.5. and 2.9.6.).

2.9.1. Rho GTPases and regulation of reactive oxygen species (ROS)

Reactive oxygen species (ROS) are generated in living organisms when exposed to external factors like radiation, ultraviolet, metals and toxic substances. ROS are also produced in mitochondria as a by-product of aerobic respiration. So called "oxidative stress" occurs if ROS reach abnormally high levels. ROS react with various intracellular components such as DNA, proteins, and lipids to induce damage (oxidation of nucleic acid bases, DNA strand breakage, inactivation of proteins by oxidation, lipid peroxidation). The oxidative damage to DNA (especially mitochondrial DNA (Mandavilli et al., 2002)) is also implicated in various degenerative diseases, cancers, and aging.

However, specific signaling mechanisms can also induce the production of ROS during the innate immune response to pathogens or during wound healing. Phagocytic leukocytes generate high concentrations of ROS to kill invading bacteria during inflammation. Thereby the cells produce ROS via a membrane-associated reduced nicotinamide adenine dinucleotide

phosphate (NADPH) oxidase. This multicomponent enzyme utilizes electrons derived from intracellular NADPH to generate superoxide anion ($\cdot O_2^-$), which subsequently dismutes to hydrogen peroxide (H_2O_2) and further may be converted into the very toxic hydroxyl radicals ($\cdot OH^-$) by iron or copper ions. Hydroxyl radicals and other ROS can then be used for host defense.

Remarkably, NADPH oxidase is one of the best-characterized Rac GTPase-regulated systems. Mammalian Rac1 and especially Rac2 have been shown to promote the assembly of the NADPH oxidase subunits (reviewed by (Diebold and Bokoch, 2005). Cdc42, on the other hand, seems to behave as an antagonistic competitor of Rac for binding to one of the subunits of NADPH oxidase (Diebold et al., 2004). These Rho GTPases are implied in the regulation of a large variety of important cellular processes (see Chap. 1.2.5.). In phagocytic leukocytes, Rho GTPases have evolved roles as crucial regulators of chemotaxis, polarized movement and phagocytosis during the innate immune response. Thus, the ability of Rac to stimulate superoxide production via the NADPH oxidase reflects just one mechanism how Rho GTPases regulate the innate immune response. Intriguingly, Rac dependent NADPH oxidase is localized at the leading edge of migrating cells. There the locally produced ROS is required for cytoskeletal reorganization and directed cell migration (reviewed by (Ushio-Fukai, 2006).

Interestingly, dominant negative forms of both Rac and Cdc42 have been reported to inhibit the production of ROS (Diebold et al., 2004; Irani et al., 1997; Sundaresan et al., 1996). The inhibition appears to occur in both cases through direct binding to one subunit (cytochrome b558) of the NADPH oxidase (Diebold et al., 2004). Consistent with these findings, expression of constitutive active Rac increases ROS levels (Sulciner et al., 1996).

Thus, although Rac and Cdc42 have antagonistic roles in NADPH oxidase regulation, the expression of dominant negative forms of both Rac and Cdc42 probably results in lower ROS levels. The tester fly lines we used in our EP screen also express dominant negative forms of either Drac1 ($Drac1^{N17}$) or Dcdc42 ($Dcdc42^{N17}$). Thus, the rough eye phenotype of these tester lines could be, at least partially, a cause of lowered ROS levels in the affected cells. Therefore, one possible mechanism for suppression of the $Drac1^{N17}$ or $Dcdc42^{N17}$ phenotypes is a direct regulation of ROS levels.

How can low ROS levels lead to a rough eye phenotype? In addition to their role in bacterial killing by leukocytes, ROS generated by NADPH oxidase have been increasingly recognized as important components of signaling in other cell types. It has been shown that signal transduction from membrane receptors of various growth factors, cytokines, or other ligands is enhanced by ROS (Bae et al., 1997), reviewed by (Rhee, 2006). Furthermore, by triggering concomitantly the activation of NADPH oxidases these receptors can induce positive feedback effects on signal

transduction. ROS mediated signaling can have positive effects on cell proliferation (Boonstra and Post, 2004). Moreover, cancer cells often have high levels of ROS (Szatrowski and Nathan, 1991).

Consistently, overexpression of catalase (Brown et al., 1999) and MnSOD (Zhong et al., 1996) leads to inhibition of cell proliferation. These two enzymes catalyze a specific ROS, H_2O_2, into oxygen and water and hence lower the levels of ROS. Furthermore, eye-specific overexpression of the transcriptional activator of catalase, DREF (see also below Chap. *dTrf2*), leads to a rough eye phenotype which can be suppressed by an amorphic catalase allele. DREF is known to play an important role in regulating cell proliferation-related genes (Hyun et al., 2005; Yamaguchi et al., 1995). Thus, the rough eye phenotype could be a cause of disregulated cell proliferation. Therefore, the identified suppressor genes could interfere with cell proliferation. Interestingly, in addition to the six genes proposed to have a function in ROS regulation, two further genes identified in the EP screen possibly fit into this group of genes since they are known regulators of cell proliferation: *Rbf* and *string* (see below). Several reports have shown that ROS and NO (see below) affected Rbf and cdc25 function (Buhrman et al., 2005; Bulavin et al., 2001; Douglas et al., 2005; Douglas et al., 2001; Kuzin et al., 2000; Rudolph, 2005; Zhang et al., 2003b).

On the other hand, high levels of ROS can induce cell death by activating apoptosis specific pathways (Boonstra and Post, 2004). This makes sense since thereby cells that potentially have accumulated DNA mutations induced by ROS can be eliminated.

The expression of both dominant negative Drac1 and Dcdc42 GTPases is suggested to result in NADPH oxidase inhibtion and consequently in low ROS levels. Therefore, suppressor genes should somehow increase ROS levels or imitate the signaling effects of ROS. However, as discussed below, some of the identified genes with a suggested role in ROS signaling rather act negatively on ROS production. Furthermore, the fact that the suppressors interacted specifically with only either one of the two tester lines remains to be explained.

2.9.2. Suppressors with possible role in ROS homeostasis/signaling

Drosophila TBP related factor 2 (dTrf2)

Drosophila TATA-box-binding protein (TBP)-related factor 2 (TRF2) is a member of a family of TBP-related factors present in metazoan organisms. However, unlike TBP, it fails to bind to DNA containing canonical TATA boxes. Since TRF2 is associated with chromosome loci distinct from TBP, it may have different promoter specificity and regulate a subset of genes (Rabenstein et al., 1999).

A *Drosophila* complex containing Trf2 has been identified by antibody affinity purification. This

complex contains components of the nucleosome remodelling factor (NURF) complex as well as the DNA replication-related element (DRE)-binding factor DREF (Hochheimer et al., 2002).

The *Drosophila* NURF complex is a chromatin remodeling complex that catalyzes nucleosome repositioning at promoter regions to regulate access by the transcription machinery. Interestingly, it has been reported that the direction of NURF-induced nucleosome movement can be significantly modulated by GAL4 sites (Kang et al., 2002). Therefore it seems possible that dTrf2 causes suppression by an interaction with the UAS/Gal4 system rather than by a specific interaction with Dcdc42. However, the fact that dTrf2 specifically suppressed the sev>cdc42^{N17} phenotype but not the phenotypes of other tester lines (Tab. 2) does not support the notion of a general inhibition of the UAS/Gal4 system by dTrf2.

Interestingly, Trf2 bound as well to DREF. DREF is a transcription factor that specifically binds to the promoter-activating element DRE (DNA replication-related element) (Hirose et al., 1996). The DRE/DREF system plays an important role in transcription of genes related to cell proliferation, such as PCNA (proliferating cell nuclear antigen), DNA polymerase, CycA, D-raf, and E2F (Hirose et al., 2001; Ohno et al., 1996; Ryu et al., 1997; Yamaguchi et al., 1995). DREF also regulates the expression of *catalase* (Park et al., 2004) and *sps2* (Jin et al., 2004). Both genes are involved in protection of the cell to harmful ROS concentrations (Burk, 1990; Morey et al., 2003b). The fact that a rough eye phenotype caused by ectopic DREF expression can be suppressed by a *catalase* mutation (Park et al., 2004) further indicates that these two genes interact with each other. This suggests that DREF and Trf2 not only regulate general cell proliferation genes but also DNA damage protection genes. This is consistent with PCNA being also an essential protein for repair of DNA damage (Essers et al., 2005).

DREF/Trf2 activate the expression of catalase and sps2. The products of these two genes lower the levels of ROS. As mentioned above, Dcdc42^{N17} was reported to inhibit NADPH oxidase and its expression should therefore also lead to lower ROS levels. The suppression by *dTrf2* overexpression rather suggests the contrary.

Since DREF and Trf2 were found in a complex together with the chromatin remodeling NURF components it is interesting to note that several trithorax group genes with a role in chromatin remodeling interacted as well with the DREF rough eye phenotype (Hirose et al., 2001). One of these interacting genes, *moira,* was also identified in the EP screen. However, *moira* overexpression (in the EP line EP09-012) not only suppressed the sev>Dcdc42^{N17} phenotype but also the phenotypes of other tester lines (see Chap. 2.4., Tab. 2 and 3, and data not shown). Therefore we discarded this EP line.

A further role for Trf2 in cell cycle and G2-M checkpoint regulation was suggested (Shimada et al., 2003). Thus, Trf2 could act as a checkpoint regulator to assure that damaged DNA gets

repaired before progression of the cell cycle.

In summary, DREF and its co-factor Trf2 regulate transcription of genes that are involved in cell proliferation and DNA replication. This regulation possibly happens in a bigger complex that contains the chromatin remodeling NURF components as well as gene products of the trithorax group. Furthermore, the fact that *catalase, sps2* and PCNA are target genes of DREF and Trf2 indicates that they also regulate at least some aspects of the antioxidant defense system and the repair of DNA damage (possibly caused by excessive ROS).

However, it remains unclear how exactly Trf2 and Dcdc42 genetically interact. A possible involvement of Dcdc42 in G2-M checkpoint regulation has already been suggested (Muris et al., 2002; Richman et al., 1999) and is discussed further below in chapter 2.9.4. (*string* and *Rbf*). Thus, it is possible that Dcdc42 and Trf2 act in the same pathway to control progression of the cell cycle.

SelD

selD is the *Drosophila* ortholog of the human selenophosphate synthetase 1 (*sps1*) gene. Like the Trf2-DREF target gene *sps2* (see above), *sps1* is involved in the biosynthesis of selenoproteins. Since selenoproteins often have important antioxidant functions by neutralizing ROS they affect as well the intracellular ROS levels (Alsina et al., 1999; Burk, 1990; Morey et al., 2001). Accordingly, a null mutation in *selD* causes an impairment of selenoprotein synthesis and a strong increase in intracellular ROS (Morey et al., 2003a). Consistently, *selD* mutant flies are hypersensitive to oxidative stress (Morey et al., 2003b).

Furthermore, *selD* mutant flies have a higher proportion of cells that are arrested at the G2 phase of the cell cycle (Alsina et al., 1999) and cell proliferation and differentiation are abnormal (Alsina et al., 1998; Serras et al., 2001).

This suggests that the Drac1^{N17}-mediated rough eye phenotype is at least partly caused by impaired ROS signaling and possibly an altered cell cycle checkpoint regulation.

However, although *selD* overexpression in EP36-054 led to suppression of the GMR>Drac1^{N17} phenotype, no interaction with the sev>Dcdc42^{N17} phenotype was observed. On the other hand, the transcriptional activator of *sps2,* Trf2 (EP13-034, see above), was identified exclusively in the screen for suppressors of the sev>Dcdc42^{N17} phenotype. Therefore, it is difficult to imagine how both *Trf2* and *SelD* control similar aspects of ROS signaling since they interact exclusively with one and not both of the small GTPases.

ATP7

ATP7 encodes a P-type transmembrane ATPase. It is the single ortholog of the mammalian Menkes (ATP7A; MNK) and Wilson (ATP7B; WND) disease proteins (reviewed by (Voskoboinik and Camakaris, 2002; Voskoboinik et al., 2002). These proteins serve a dual function. They deliver copper ions to cuproenzymes in the Golgi compartment and translocate excess copper outside the cell (Pase et al., 2004). Indeed, accumulation of copper in *Drosophila* S2 cells was significantly increased when *ATP7* was suppressed using double-stranded RNA interference, demonstrating that ATP7 is essential for efflux of excess copper (Southon et al., 2004).

Menkes disease in humans is a rare neurological disease that manifests in progressive neuronal degeneration and death in early childhood. The defects arise from impaired cellular efflux and thus excessive copper accumulation in the cells. Besides being an essential metal ion for the function of key metabolic enzymes, copper has a pro-oxidant activity and can therefore be a source of ROS. Since the central nervous system has a high oxygen consumption and relative poor antioxidant defense, it is highly susceptible to ROS-mediated toxicity. Thus, it has been proposed that oxidative damage would be the main cause for the degeneration of neuronal cells in Menkes disease patients (reviewed by (Llanos and Mercer, 2002; Rossi et al., 2004).

Interestingly, a recent report implicated the Rho GTPase Dcdc42 in regulating the trafficking of Menkes disease protein between different membrane compartments (Cobbold et al., 2002). Thus, it is tempting to speculate that expression of $Dcdc42^{N17}$ leads to mislocalization of ATP7, which in turn results in unsufficient efflux of intracellular copper. The excess copper then causes oxidative damage through production of ROS. This would be rescued by overexpression of ATP7, which is obtained with the identified EP element insertion. Indeed, it has been shown in mammalian cell culture studies that enhanced expression of ATP7A was sufficient to correct the copper accumulation and copper retention phenotype of ATP7 mutant cells (Camakaris et al., 1995; La Fontaine et al., 1998).

However, as mentioned above, $Dcdc42^{N17}$ was reported to inhibit NADPH oxidase and its expression should therefore lead to lower ROS levels. The suppression by *ATP7* overexpression rather suggests the contrary.

CG6700

The protein encoded by CG6700 contains at its C-terminus a SAC3/GANP domain. Proteins sharing this motif are involved in nuclear export and mitotic progression (Bauer and Kolling, 1996; Jones et al., 2000; Khuda et al., 2004; Lei et al., 2003). Interestingly, the human homolog LENG8 was suggested to be involved in host defense response to pathogens by leukocytes since the gene lies in the leukocyte receptor cluster (LRC) located in the 19q13.4 region that contains mainly genes encoding killer cell inhibitory receptors (KIR) and immunoglobulin-like

transcripts (ILT) (Wende et al., 1999). Such genes do not lie in the vicinity of CG6700. However, located next to CG6700 is another gene for which a role in the defense response has been reported: NOS, the gene that encodes the nitric oxide (NO) synthase. An attractive, but nevertheless very speculative model would consider CG6700 and NOS as two clustered genes that become activated upon attack of pathogens. Alternatively, NOS itself could be the gene responsible for the suppression phenotype since the last exon of CG6700 overlaps with the first exon of NOS. Thus, the EP element EP43-011 could drive both CG6700 and NOS upon Gal4-activation.

NO, the product of NOS, is a free radical that is very reactive and unstable, like ROS. For example, it reacts with superoxide to form peroxynitrite (ONOO-), a reactive nitrogen species (RNS) that can oxidize and thereby damage DNA, proteins and lipids. On the other hand, again like ROS, in moderate levels NO can act as a signaling molecule to induce gene expression, cell differentiation and immune activation (Foley and O'Farrell, 2003; Kuzin et al., 2000; Kuzin et al., 1996).

Interestingly, yeast two-hybrid and GST pull-down assays revealed both Rac1 and Rac2 small GTPases as NOS interacting proteins (Kuncewicz et al., 2001). Furthermore, the dominant negative $Rac1^{N17}$ was shown to suppress expression of NOS in rat livers (Harada et al., 2003).

Also, NO was shown to have an important role in wound healing (reviewed by (Luo and Chen, 2005; Rizk et al., 2004). A recent study could show that increased ROS levels caused nitric oxide deficiency and delayed wound healing. This effect could be suppressed by expression of *Rac1*^{N17} (Luo et al., 2004). Thus, NO signaling seems to interact with ROS signaling.

In *Drosophila*, NO has been reported to regulate cell proliferation in imaginal discs (Kuzin et al., 1996). Interestingly, ectopic expression of *NOS* in the *Drosophila* eye suppressed the rough eye overexpression phenotype of *dE2F*, and *Rbf* interacted synergistically with *NOS* (Kuzin et al., 2000). In the course of the EP screen, overexpression of *Rbf* was also found to suppress the *Dcdc42*^{N17} rough eye penotype (see below). This is important since the EP line 43-011 that drives *CG6700* and possibly *NOS* was also identified as suppressor of the *Dcdc42*^{N17} but not of the *Drac1*^{N17} phenotype. Thus, despite the well documented interactions between NOS and Rac, especially in the context of wound healing, here NOS would probably interfere with cell proliferation. The role of CG6700 itself remains to be analyzed.

6-phosphofructo-2-kinase (pfrx)

pfrx encodes the glycolytic enzyme that catalyzes the irreversible transfer of a phosphate from ATP to fructose-6-phosphate. Since this step is irreversible, pfrx (PFK in mammals) gets a key role in the control of glycolysis.

In *Drosophila*, *pfrx* shows strong expression in crystal cells. These cells are part of the *Drosophila* hematopoietic system. Together with plasmatocytes and lamellocytes, they provide the organism with the capacity for wound healing, immune response, and removal of apoptotic cells.

There is some evidence from mammalian cell culture studies that the Rac/Cdc42 effector Pak regulates the glycolytic pathway in a related cell type, phagocytes, during host defense response (Shalom-Barak and Knaus, 2002). Thus, the $Rac1^{N17}$ phenotype could be a result of disregulated glycolysis that would be suppressed by enhanced *pfrx* expression.

Interestingly, most of the glycolytic enzymes, including phosphofructokinase, have been reported to bind to the actin and tubulin cytoskeleton (Knull and Walsh, 1992). This interaction has been identified as an important mechanism to generate ATP in the vicinity of the cytoskeleton and thereby modulating cell morphology. However, it remains difficult to imagine how the mere overexpression of *pfrx* can rescue the $Drac1^{N17}$ phenotype.

Another possible mechanism for the suppression of $Rac1^{N17}$ by *pfrx* expression could involve again ROS since high levels of glycolytic products can induce ROS (at the mitochondria). High extracellular glucose levels (hyperglycemia) also have been reported to augment intracellular ROS levels through several ways ((Nishikawa et al., 2000), reviewed by (Brownlee, 2001)). Interestingly, ROS formation induced by this extracellular high glucose could be inhibited by expression of dominant negative Rac1 ($Rac1^{N17}$) (Luo et al., 2004). The authors also showed that the augmented ROS levels led to NO deficiency and consequently to impairment of wound healing. NO in turn has been shown in another study to suppress PFK activity (Tsuura et al., 1998) and furthermore, PFK has been found to be associated with NOS in an affinity chromatography assay (Firestein and Bredt, 1999).

The clear link between *pfrx* and *Drac1* still remains to be established. It might be interesting to further evaluate the role of *pfrx* in wound healing and its connection to ROS, NO and especially Rac signaling.

CG30421

The protein encoded by CG30421 contains a ubiquitin carboxyl-terminal hydrolase family 2 (UCH2) domain. This domain is shared by enzymes that cleave off ubiquitin residues from proteins and thereby are likely to inhibit ubiquitin dependent degradation by the proteasome complex (Amerik and Hochstrasser, 2004).

The increasing number of deubiquitinating enzymes raises the possibility that specific protein turnover rates can be differentially regulated by these enzymes. Such regulation would imply that deubiquitinating enzymes possess a considerable degree of substrate specificity.

Interestingely a Y2H screen database (http://pim.hybrigenics.com) recently reported an interaction of CG30421p with the small GTPase Rap1 (also known as Roughened and Dras3). This raises the possibility that CG30421 acts as a specific deubiquitinating enzyme of small GTPases. Thus the prevention of degradation of the endogenous GTPase Dcdc42 may be responsible for the suppression phenotype. Rap1 has been previously reported to interact with another deubiquitinating enzyme (fat facets) in eye development (Li et al., 1997).
Moreover Rap1 has been shown to have a function in DC (Boettner et al., 2003) which additionally supports a function of CG30421 in DC. Rap1 is involved in the regulation of both integrin- and cadherin-mediated cell adhesion in several cases (Caron et al., 2000; Fujita et al., 2005; Hogan et al., 2004; Huelsmann et al., 2006; Reedquist et al., 2000). Interestingly, Cdc42 has been suggested to function downstream of Rap1 in mammalian epithelial cell cultures and in yeast cells (Chant and Stowers, 1995; Hogan et al., 2004). Rap1 is also a highly abundant protein in phagocyte membranes and Huelsmann et al. (2006) showed that it regulates integrin-dependent adhesion and migration (Huelsmann et al., 2006). Additionally, Rap1 has been reported to be a subunit of NADPH oxidase (Vignais, 2002), and expression of wild type or dominant negative as well active forms of Rap1 affects ROS production (Gabig et al., 1995). This suggests the further possibility that the gene product of CG30421 affects ROS signaling via Rap1 or even Dcdc42.

2.9.3. Summary of suppressor genes with a proposed function in ROS signaling

Cdc42 and especially Rac have well documented roles in the regulation of NADPH oxidase. The analysis of the genes that were identified in the EP screen suggests that the Rho GTPases regulate ROS homeostasis not only through NADPH oxidase but also through other mechanisms. The proposed inhibition of NADPH oxidase and therefore downregulation of ROS by the Dcdc42^{N17}/Drac1^{N17}-expression in the tester lines should lead to lowered ROS levels. However, EP dependent overexpression of three suppressor genes (*dTrf2, dATP7A* and *SelD*) probably leads itself to lowered ROS levels. This rather suggests that expression of DN Drac/Dcdc42 in fact leads to elevated levels of ROS. On the other hand, EP dependent overexpression of the suppressor gene *pfrx* (and possibly also CG6700 or *NOS*) probably leads to elevated ROS. In the case of CG30421 it is not apparent how ROS levels would be affected. Thus, although these six genes potentially are involved in ROS signaling, their exact roles remain to be assessed.
The effects of Dcdc42^{N17} or Drac1^{N17}-expression on ROS levels and the role of the six genes in

this context could be tested by several means: First, as in *SelD* mutant clones of *Drosophila* eye imaginal discs (Morey et al., 2003a), ROS could be detected *in vivo* in the Dcdc42^{N17}/Drac1^{N17}-tester lines. The ROS levels could then be compared to those in flies carrying additionally a suppressing EP element. Second, Dcdc42^{N17}/Drac1^{N17} and EP-dependent expression could be tested whether it may confer to sensitivity to oxidative stress (e.g. paraquat or hydrogen peroxide) (Morey et al., 2003b). Third, antioxidants or specific inhibitors of the NADPH oxidase (Diebold et al., 2004) could be tested whether they modify the overexpression phenotypes. To further support the relevance of the potential connection between ROS, Rho GTPase signaling and the identified six genes, such experiments should be performed also with loss-of-function mutants.

A schematic representation of the six genes in the context of ROS signaling is shown in Figure 16 (Chap. 4.2.). Rbf and string and a further identified suppressor, Falafel (see below), are as well represented in Figure 16 because of their roles in cell cycle checkpoint regulation and DNA damage repair in response to various stresses, including ROS.

2.9.4. *string* and *Rbf*, two suppressors with a role in cell cycle regulation, possibly dependent on ROS levels?

As discussed above, ROS can damage several cell components but can also act as a signaling molecule. ROS mediated DNA damage leads to cell cycle arrest, thus it makes sense that cell cycle regulators are influenced by ROS. Two suppressors that have been identified in the Dcdc42-screen correspond to well known cell cycle regulators: *string* (cdc25) and *Rbf* (see below). This finding further supports the role of Cdc42 in cell cycle checkpoint regulation in response to ROS.

string is a Cdc25-type phosphatase that promotes cell cycle progression. In a wild-type background, string is required for progression from G2 to mitosis; when overexpressed it causes premature initiation of mitosis (Edgar and O'Farrell, 1989).

string has been targeted by another independent EP insertion and was also identified to suppress the phenotype of the tester line sal-Gal4>UAS-lgs^{17E} that was designed to identify wingless signaling components. Thus, string is not a specific suppressor of Dcdc42^{N17}. Interestingly, twist and zerknüllt are two transcription factors shown to alter string expression and they are regulated by dpp signaling in early embryogenesis. One might speculate that Dcdc42 signaling downstream of dpp induces twist and zen expression and these in turn activate string expression. Thus, downregulated cdc42 signaling could be restored by string expression.

Surprisingly, Rbf was also identified in the sev>Dcdc42^{N17} screen. Whereas string promotes cell cycle progression from G2 to M phase, Rbf is known to block transition from G1 to S phase. Recently, the human ortholog of Rbf (Rb) was also shown to be involved in the G2/M transition. Disruption of RB accelerated G2/M progression in the presence of DNA damage by elevating the expression of a set of mitotic regulatory genes (Eguchi et al., 2006). Thus, the simplest explanation for the interaction of cdc42 with both string and Rbf could be that Dcdc42^{N17} expression leads to an abnormally high level of G2 arrested cells which would be suppressed by string or Rbf overexpression. In yeast there is evidence that Cdc42 is indeed essential for G2/M transition (Richman et al., 1999). On the other hand, it has been shown in mammalian cells that Cdc42 can inhibit cell cycle progression at G1/S through a mechanism requiring activation of p38 (Molnar et al., 1997). Thus, the inhibition of G1/S progression by Dcdc42 could be prevented by expression of Dcdc42^{N17}, eventually leading to more cells arrested in G2.

I observed a clear reduction of bristles on the thorax upon *stg* or *Rbf* overexpression (Fig. 8 E (*Rbf*) and data not shown). Pena-Rangel et al. (2002) reported the same phenotype when *stg* was overexpressed in the thorax (Pena-Rangel et al., 2002). This phenotype might be explained by the fact that exit from the cell cycle into G2 is required for proper neural cell fate determination (Negre et al., 2003). This exit would be perturbed by both *stg* or *Rbf* overexpression.

The G2 checkpoint prevents cells from entering mitosis when DNA is damaged, providing an opportunity for repair and stopping the proliferation of damaged cells. ROS are known to damage DNA. Interestingly, the mammalian homolog of string, cdc25, has been shown to be regulated by ROS (Savitsky and Finkel, 2002). The mechanism for this regulation is explained by the fact that cdc25 has at its active site a highly reactive cystein-disulfid-bond that can react directly with ROS, leading to enzyme inactivation (reviewed by(Rudolph, 2005). A role for cdc25 in DNA damage response is further supported by several evidences that place cdc25 downstream of ATM and Chk2 (reviewed by (Busino et al., 2004).

Considering the herein proposed new function/regulation of cdc42 and Rac by ROS signaling, it is worthwhile to speculate that cdc42 function in cell cycle regulation is also sensitive to ROS.

2.9.5. Remaining unclassifiable suppressors of the Dcdc42^{N17}-phenotype

glutamyl-prolyl-tRNA synthetase (Aats-Glupro)

Aats-Glupro catalyses the aminoacylation of glutamic acid and proline tRNA, respectively (Cerini et al., 1997). It is difficult to imagine how a component of the tRNA synthetase machinery can specifically regulate Dcdc42 signaling.

enhancer of yellow 3 (e(y)3)

The protein encoded by *enhancer of yellow 3, e(y)3*, contains an AT-hook, two PHD fingers, and a novel evolutionarily conserved domain with a transcriptional coactivator function. The function of the PHD finger motif is not yet known but it has been suggested to be involved in the regulation of transcription by direct binding of the DNA as well as in protein-protein interaction. BLAST homology searches for e(y)3 reveal only proteins of unknown function except for a short stretch of homology to the vertebrate transcription factor Requiem which is required for apoptosis. To further investigate the function of e(y)3 loss-of-function analysis should be performed. Several recessive lethal P-element insertions in e(y)3 are available at the Bloomington stock center. The mutant flies die in the late pupal stage as pharate adults. Two reports corroborate an essential function for e(y)3 during embryonic development (Nikolenko Iu et al., 2005; Shidlovskii et al., 2005). They also show that e(y)3 acts indeed as a transcriptional coactivator. The possible specific function of e(y)3 in the context of Dcdc42 signaling has to be further investigated.

CG30188

CG30188 encodes a protein that contains two immunoglobulin-like domains. BLAST searches do not reveal homologous proteins with an already known function. CG30188 showed no overexpression phenotypes with the tested driver lines. No mutant lines are available at the public stock centers. The long distance (13234 bp) between the insertion site of the EP element and the translation start site of CG30188 would make it difficult to generate deletion mutations. Therefore I decided not to work further on this line.

2.9.6. Remaining unclassifiable suppressors of the Drac1^{N17}-phenotype

HLHm7

HLHm7 encodes a small basic helix-loop-helix (bHLH) protein that belongs to eight partially redundant genes of the Enhancer of split complex, E(spl)-C. Notch activates the E(spl)-C genes which, in a negative feedback loop, negatively regulate expression of the Notch ligand Delta through the achaete-scute complex genes (Heitzler et al., 1996).

These genes act downstream of Notch to repress the adoption of neural and other differentiated cell fates. This happens through a process known as lateral inhibition whereby a single cell in a cluster of neurocompetent cells becomes neural while the other cells remain undifferentiated.

The overexpression phenotypes of HLHm7 are consistent with the fact that HLHm7 represses neural cell fate since sensory organs like interommatidial and thorax bristles are markedly reduced (data not shown).

When overexpressed in the corresponding EP lines, HLHm7 suppresses the Drac1^{N17} tester line phenotype. Drac1 and JNK signaling have been proposed to have a role in planar polarity in the Drosphila eye by affecting Notch signaling. However, this notion is solely based on overexpression data using dominant negative and active forms of Drac1. Strutt et al. (2002) provided evidence that Drac1 and JNK are not major effectors of planar polarity (Strutt et al., 2002). However, it is conceivable that the suppression of the dominant negative Drac1 (Drac1^{N17}) mediated rough eye phenotype by *HLHm7* expression is comparable to the results reported earlier concerning planar polarity and Drac1/JNK signaling (Fanto et al., 2000).

couch potato (cpo)

couch potato (cpo) encodes a *Drosophila* RNA-binding protein that has been implicated in the regulation of several aspects of proneuronal cell development (Glasscock and Tanouye, 2005). *achaete*, *scute* and *daughterless* gene functions are required for proper expression of *cpo* in the PNS (Bellen et al., 1992). *cpo* was identified as suppressor of the Drac1^{N17} phenotype. It remains to be explained how *cpo* interacts with Drac1 signaling.

CG30122

The protein encoded by CG30122 contains a SAP (after SAF-A/B, Acinus and PIAS) domain, a alpha/beta-hydrolase domain (Ollis et al., 1992), and a SPRY domain (named from SPla and the RYanodine Receptor). Furthermore there is a predicted domain COG4639 that is obtained by **c**lustering of **o**rthologous **g**roups of proteins. The SAP motif is a putative DNA binding domain found in diverse nuclear proteins involved in chromosomal organization (Aravind and Koonin,

2000). The alpha/beta hydrolase fold is common to a large group of hydrolytic enzymes of widely differing phylogenetic origin and catalytic function. The function of the SPRY domain is not known. The COG4639 sequence has a predicted function of a kinase.

CG30122 is very homologous to the vertebrate hnRNP U-like 1 (Hnrpul1), a protein that belongs to the subfamily of heterogeneous nuclear ribonucleoproteins (hnRNPs). The hnRNPs are RNA binding proteins and they complex with heterogeneous nuclear RNA (hnRNA). These proteins are associated with pre-mRNAs in the nucleus and appear to influence pre-mRNA processing and other aspects of mRNA metabolism and transport (reviewed by (Krecic and Swanson, 1999). A function for CG30122 in mRNA metabolism is supported by yeast-two-hybrid protein interactions of CG30122p with several RNA binding proteins of the spliceosome, the small nuclear ribonucleoprotein and the ribosome complexes (Fly GRID database, (Giot et al., 2003)).

What could be the connection to Rac signaling? Romero et al. detected in a yeast two-hybrid screen and with GST fusion proteins a specific interaction of the (human) Rac GDP exchange factor Vav with a heterogeneous nuclear ribonucleoprotein, hnRNP C (Romero et al., 1998). The clear role and the consequences of this interaction have not been described yet. However, the genetic data obtained with CG30122 and the physical binding between Vav and hnRNP C could reveal a new function for Rac in the regulation of at least some aspects of mRNA metabolism.

Furthermore, Hnrpul1 was identified in a complex with histones H2A, H2B, H3 and H4 (Kzhyshkowska et al., 2003). H2A is involved in the DNA damage response, it is therefore interesting to note that Hnrpul1 gets activated by p53 upon stress (Krieg et al., 2006). A role for Drac1 and one of the suppressors (Falafel) in DNA damage response and H2A regulation is discussed in chapter 3.

MSP-300/Nesprin

Only one of the two EP-elements that are inserted at the Muscle-specific protein 300 (MSP-300) gene locus is supposed to drive a full-length transcript (EP51-198) whereas EP32-120 is inserted in an intron near the putative 3' end of the gene. When the UAS sites at the 3' of EP51-198 were excised by the Cre/lox system, the suppression phenotype was lost, indicating that expression of MSP-300 is required for suppression. On the other hand however, EP32-120 still showed the suppression phenotype when the UAS sites pointing to further 3' of MSP-300 were excised by the Cre/lox system. This suggested that either the EP-element insertion EP32-120 on its own or antisense-expression of MSP-300 is responsible for the suppression phenotype. The situation is further complicated since MSP-300, an already huge protein of 7000 amino acids, has been reported to correspond to the N-terminal two-thirds of the *Drosophila nesprin* ortholog which lies further 3' (Zhang et al., 2002). The gap of 29-kb between the MSP-300 gene and the

putative *nesprin* gene is occupied by an intronless, sense-strand, 26-kb ORF ("repetitive ORF", CG31916) capable of encoding an 8722-amino-acid protein. Zhang et al. found that MSP-300/*nesprin* represents a single gene from which N- and C-terminally truncated proteins are generated, in addition to a low-abundance, full-length product of 11720 amino acids.

MSP-300 contains an amino-terminal calponin homology (CH) domain. This motif is predicted to bind actin (Gimona et al., 2002). This prediction is supported by studies that showed co-localization of MSP-300/nesprin with F-actin (Volk, 1992; Zhang et al., 2002). The central part of MSP-300/nesprin is occupied by multiple spectrin repeats. These repeats are usually found in proteins that assemble multiprotein structures involved both in cytoskeletal architecture as well as in forming large signal transduction complexes (reviewed by (Djinovic-Carugo et al., 2002). The carboxy-terminal part of MSP-300/nesprin contains a nuclear envelope localization domain called the KASH domain (for Klarsicht/ ANC-1/Syne-1 homology) (Starr and Han, 2002).

MSP-300/nesprin and homologous vertebrate proteins such as Syne-1, Syne-2 and Dystrophin, the product of the Duchenne Muscular Dystrophy gene, have assigned functions in muscle cells. MSP-300/*nesprin* is expressed by muscle precursors at muscle-ectoderm and muscle-muscle attachment sites (Rosenberg-Hasson et al., 1996; Volk, 1992). Syne-1 is similarly clustered at the neuromuscular junction and is enriched at the nuclear envelope of myonuclei. The localization to the nuclear envelope by the KASH domain and the existence of the CH actin binding domain suggest a role for these proteins in anchoring the nucleus to the cortical actin cytoskeleton. The huge size of these proteins would allow to stretch between the nucleus and the actin cytoskeleton as it was proposed for ANC-1, another KASH domain containing protein (Starr and Han, 2002). Thus, Drac1^{N17} expression could possibly disrupt the anchoring of the nucleus to the actin cytoskeleton which could somehow be restored by increased MSP-300/*nesprin* levels (with EP51-198). More generally, the actin binding protein MSP-300/nesprin is a good candidate of a Rac effector that is involved directly in the rearrangements of the cytoskeleton. It would also be important to investigate the role of Rac1 in muscle development.

Stromalin 2 (SA-2 = SNM)

Stromalin in Meiosis (SNM) was identified as a suppressor of the Drac1^{N17} tester line. It belongs to the SCC3/SA/STAG family of cohesin proteins that are required for conjunction and regular segregation of homologous chromosomes in *Drosophila* male meiosis (Thomas et al., 2005). Interestingly, the suppression phenotype was observed only in male eyes whereas the female eyes displayed an even more disrupted (enhanced) phenotype. This finding supports a sex-specific function for SNM. So far there is no evidence for Drac1 having a role in male meiosis.

CG9351/falafel (flfl)

Two EP elements (EP39-161 and EP47-082) inserted at the *falafel* locus. *falafel* encodes a novel RanBP domain containing protein that is evolutionarily conserved. Analysis of further overexpression phenotypes, subcellular localization, the generation of mutations, the analysis of the mutant phenotype (dorsal open and reduced body size) are presented in the next chapter (Chap. 3). Furthermore, an RNAi experiment against the *Caenorhabditis elegans falafel* ortholog, SMK-1, was performed. SMK-1 was shown to possess a stress responsive function by interacting with the insulin receptor (DAF-2), possibly as a co-regulator of the FOXO transcription factor DAF-16 (Wolff et al., 2006). Moreover, it was shown that the yeast and human Falafel orthologs function in a cisplatin sensitive protein phosphatase 4 (PPP4) complex (Gingras et al., 2005; Hastie et al., 2006; Wu et al., 2004)(see Chap. 3.7.2. and 3.7.3.). In collaboration with Gingras et al. (2005), we provided evidence that *Drosophila falafel* mutants also display cisplatin sensitivity (supplementary paper). These findings support a role for *falafel* in the response to DNA damage caused by various stresses.

Table 5 summarizes the molecular and functional data about all the identified suppressor genes.

RESPONSIBLE GENE	MOLECULAR AND FUNCTIONAL INFORMATION	SUPPRESSOR OF	VERTEBRATE HOMOLOG
dTrf2	TATA-box-binding protein (TBP)-related factor 2 co-regulator of DREF, involved in cell proliferation	$Dcdc42^{N17}$	TBP-like 1
ATP7	Copper-transporting P-type ATPase, required for efflux of excess cellular copper ions	$Dcdc42^{N17}$	ATP7A/B, Menkes/Wilson disease genes
CG6700	SAC3/GANP protein domain, possibly involved in nuclear export and mitotic progression	$Dcdc42^{N17}$	LENG8
pfrx	6-phosphofructo-2-kinase key enzyme in glycolysis	$Drac1^{N17}$	PFK-1

CG30421	Ubiquitin carboxyl-terminal hydrolase family 2 (UCH2) de-ubiquinating enzyme	Dcdc42^{N17}	Ubiquitin carboxyl-terminal hydrolase 43
SelD	involved in selenium-protein synthesis redox regulation, cell proliferation, imaginal disc development	Drac1^{N17}	selenophosphate synthetase 2 (sps2)
stg	protein tyrosine phosphatase regulation of G2/M transition of mitotic cell cycle	Dcdc42^{N17}	cdc25
Rbf	repressor of gene transcription tumor suppressor, pivotal role in the negative control of the cell cycle	Dcdc42^{N17}	Rb
falafel	RanBP domain, structure similar to EVH1/PH domains regulatory subunit of protein phosphatase 4 (PP4) cisplatin sensitivity ***see chapter 3***	Drac1^{N17}	PP4R3
Aats-Glupro	glutamyl-/prolyl- tRNA synthetase	Dcdc42^{N17}	glutamyl-prolyl tRNA synthetase
e(y)3	Zn-finger-like, PHD finger transcriptional coactivator	Dcdc42^{N17}	PHD finger protein 10
CG30188	Immunoglobulin-like domains cell adhesion, signal transduction?	Dcdc42^{N17}	hemicentin?
HLHm7	Basic helix-loop-helix dimerization region bHLH Enhancer of split complex, E(spl)-C Notch signaling, neuronal cell development	Drac1^{N17}	HES (Hairy and enhancer of split)
cpo	RNA-binding proneuronal cell development	Drac1^{N17}	spinocerebellar ataxia type 1 gene (SCA1) ?
CG30122	SAP domain (DNA-binding) SPRY domain COG4639 predicted kinase pre-mRNA processing?	Drac1^{N17}	Hnrpul1 (heterogeneous nuclear ribonucleoprotein U-like 1)
MSP-300/Nesprin	actin binding, nuclear envelope binding muscle development	Drac1^{N17}	Dystrophin, Nesprin, Syne
SA-2	SCC3/SA/STAG family of cohesin proteins required for meiosis	Drac1^{N17}	stromal antigen 2 (STAG2)

Table 5. Suppressor genes identified in the EP screen.

The genes with a proposed function in ROS homeostasis are yellow shaded. The two cell cycle regulators stg and Rbf are marked with red shading, and the putative DNA damage responsive gene *falafel* is orange shaded.

3. CG9351/Falafel: A novel regulator of dorsal closure

3.1. Identification of the gene responsible for suppression

Two independent EP element insertions (EP39-161 and EP47-082) were located at almost the same site, at a distance of 10 bp to each other, in the 5' UTR of CG9351 (Fig. 10). In the following I will refer to this gene as *falafel*, based on the mutant phenotype (see Chap. 3.6.). Both EP lines displayed a very similar suppression phenotype when crossed to the Drac1^{N17}

tester line (Fig. 7, Chap. 2.4.). CG9591, a gene with unknown function so far, lies 5' to the two EP elements. A further single-headed EP element line driving Gal4-mediated expression of CG9591 (EP(3)0929, (Rorth, 1996)) was available from public stock centers and tested for suppression of the Drac1^{N17} phenotype. No suppression was observed indicating that CG9591 was not the responsible gene.

Cre-mediated excision (see Chap. 2.6.2) of the UAS-containing sequences on the 5' part of the EP element EP47-082 led to a single-headed EP element with UAS directed only to *falafel* (EP47-082(loxed)). This EP element still showed suppression of the Drac1^{N17} tester line phenotype indicating that *falafel* was the responsible gene. Moreover, a UAS-*falafel* construct was generated and transgenic flies were obtained that showed the same suppression phenotype. These findings clearly show that *falafel* is the gene responsible for suppression of the rough eye phenotype of the Drac1^{N17} tester flies.

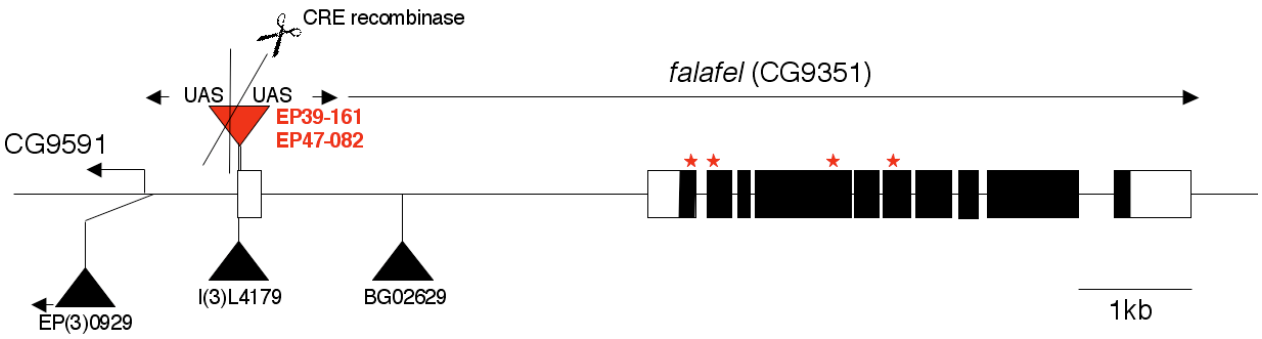


Figure 10. Gene locus of *falafel* and P-element insertion sites.

The red triangle marks the insertion site of the two EP elements EP39-161 and EP47-082, which is in the 5'UTR (left white box). The black boxes designate the translated transcript. Another P-element, l(3)L4179, is inserted at the very same position as EP47-082. Red asterisks mark the positions of mutations in *falafel* that were obtained following mutagenesis (see Chap. 3.5.). The EP element EP(3)0929 with UAS sequences directed to CG9591 is located 800 bp further upstream in the 5'UTR of CG9591.

3.2. Further overexpression phenotypes of *falafel*

The rough eye phenotype of GMR-Gal4>UAS-Drac1^{N17} flies was suppressed by co-overexpression of *falafel* (Fig. 11 A).

When crossed to different Gal4-drivers alone, EP39-161 and EP47-082 produced several misexpression phenotypes, including wing defects, a strong thorax cleft and pupal lethality (Fig. 11), depending on the Gal4 driver line used.

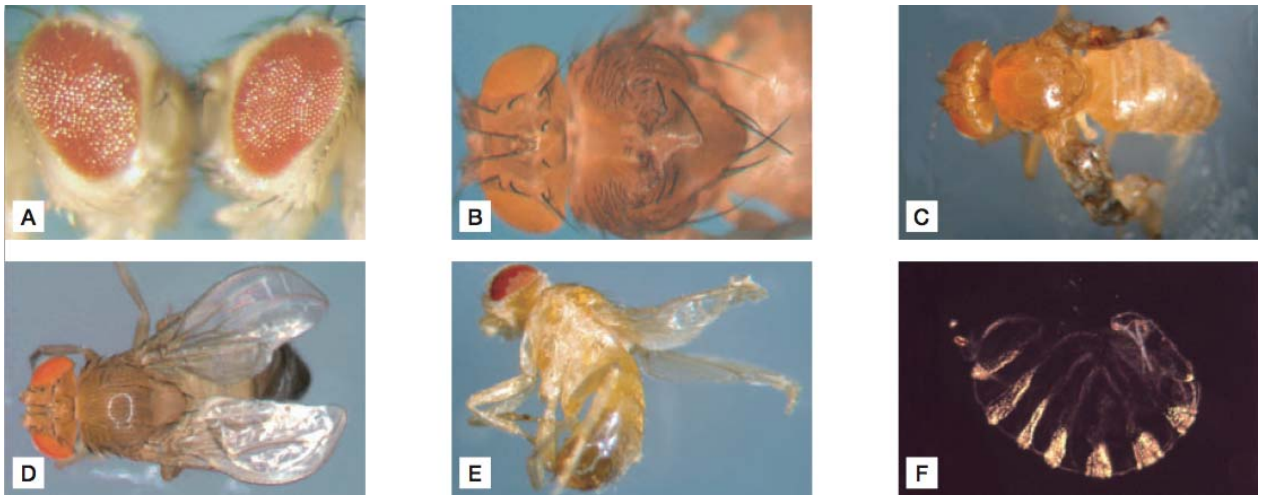


Figure 11. Overexpression phenotypes of *falafel*

(A, left side) Rough eye phenotype of the GMR>DRac1^{N17} tester line, (A, right side) suppression by co-overexpression of *falafel* in the EP47-082 line. (B) Thorax cleft phenotype in *pnr^{MD237}*>UAS-*falafel* flies. (C) Wing (but no thorax) defect in apGal4>UAS-*falafel* flies. (D) Defective posterior wing parts in *en*Gal4>UAS-*falafel* flies. (E) Wing defects in 69B>EP47-082(loxed) flies. (F) Dorsal closure defects in 69B>EP47-082 embryos.

The MD237 Gal4 enhancer trap insertion insertion in the *pannier* locus (*pnr^{MD237}*) drives expression in the mediodorsal parts of thoracic and abdominal segments of embryos, larvae and adult flies (Calleja et al., 2000). Together with one of the two EP elements, EP39-161 or 47-082, MD237 gives rise to a penetrant thorax cleft phenotype (Fig.11 B).

Thorax closure during pupariation resembles the process of dorsal closure during late embryogenesis. In addition to morphological similarities, genetic evidence points to similar

molecular mechanisms, led by the JNK and Dpp signaling pathways, directing embryonic dorsal and imaginal thorax closure (Agnes et al., 1999; Martin-Blanco et al., 2000; Zeitlinger and Bohmann, 1999).

However, crossing the EP elements with *ap*Gal4 (Calleja et al., 2000), which drives expression in a similar domain as pnr^{MD237}, does not result in a thorax cleft but in wing defects (Fig. 11 C). This suggests that the thorax cleft may be caused by genetic interaction of *falafel* overexpression with pnr^{MD237}, which is itself also a hypomorphic *pnr* allele (Calleja et al., 2000; Pena-Rangel et al., 2002).

Overexpression of *falafel* by using an *engrailed*–Gal4 enhancer trap line led to another specific wing phenotype. In these wings the posterior *engrailed*-Gal4 expressing part was markedly reduced (Fig 11 D).

Wing defects are also obtained with the P{GawB}69B enhancer trap line (see Fig. 8, Chap. 2.5.). When using the single-headed EP47-082(loxed) the wing defect is more subtle. The distal portion of the wing blade of 69B>EP47-082(loxed) flies is folded back (Fig. 11 E). This Gal4 line drives expression of UAS in the embryonic ectoderm from stage 11 and in the eye-antennal, haltere, leg and wing imaginal discs. Apart from the specific wing phenotype, no other adult tissues appear to be affected in 69B>EP47-082 flies. Nevertheless, embryogenesis also seems to be impaired to some extent since several dead embryos were observed and examined. These embryos displayed variable phenotypes including anterior open, dorsal open and germ band retraction defective phenotypes (Fig. 11 F).

armadillo-Gal4 (arm-Gal4, P{GAL4-arm.S}) (Rorth et al., 1998) is a weak Gal4 line that drives ubiquitous expression throughout development. *falafel* overexpression by arm-Gal4 gave rise to healthy flies with slightly corrugated wings (not shown).

The strong ubiquitously expressed Gal4 driver line Act5CGal4 (Ito et al., 1997) gave rise to early pupal lethality when crossed to either EP39-161 and 47-082 (not shown).

JNK signaling target gene expression upon overexpression of *falafel* was examined. For this purpose, *puc*LacZ reporter gene expression was followed in embryos that overexpress CG9351 (in EP47-082) under the control of the pnr^{MD237} Gal4 driver. *puc*LacZ expression was not altered in pnr^{MD237}>EP47-082 embryos indicating that JNK target gene expression may not be affected by *falafel* overexpression (data not shown).

3.3. Falafel is a novel well conserved RanBP domain containing protein

falafel encodes a novel evolutionarily conserved protein. Figure 12 A shows an CLUSTAL W alignment (Thompson et al., 1994) of the protein sequences of Falafel, one of the human

orthologs, PP4R3α, the *C.elegans* ortholog SMK-1, and the budding yeast ortholog PSY2. Falafel shares significant identity and similarity at the amino acid level in the aligned region with PP4R3α (62% identity and 83% similarity), SMK-1 (40%/64%) and Psy2 (27%/46%). The RanBP domain is the most conserved portion of the protein across species. For example, the RanBP domains of Falafel and PP4R3α share 87% identity and 95% similarity.

Figure 12 B shows a schematic representation of the Falafel protein domains. The predicted two- and three-dimensional structures for a fragment of 120 amino acids at the N-terminus show an extensive structural homology to the Ran binding domain found in RanBP2 (Gingras et al., 2005). Interestingly, many of the amino acid residues establishing contact with Ran are conserved. This raises the intriguing possibility that this portion of Falafel could serve as a small GTPase-binding domain. The human ortholog of Falafel, PPP4R3, was tested for interaction with Ran by co-immunoprecipitation assays, but no binding could be detected (Gingras et al., 2005).

The predicted structure of this N-terminal fragment also shows homology to pleckstrin homology (PH) and enabled/VASP (vasodilator-stimulated protein) homology 1 (EVH1) domains.

The PH domain occurs in a large variety of signaling proteins. It has been demonstrated to recognize phosphoinositide headgroups (Lemmon, 2003) and can thereby localize to the plasma and internal membranes (Cozier et al., 2004).

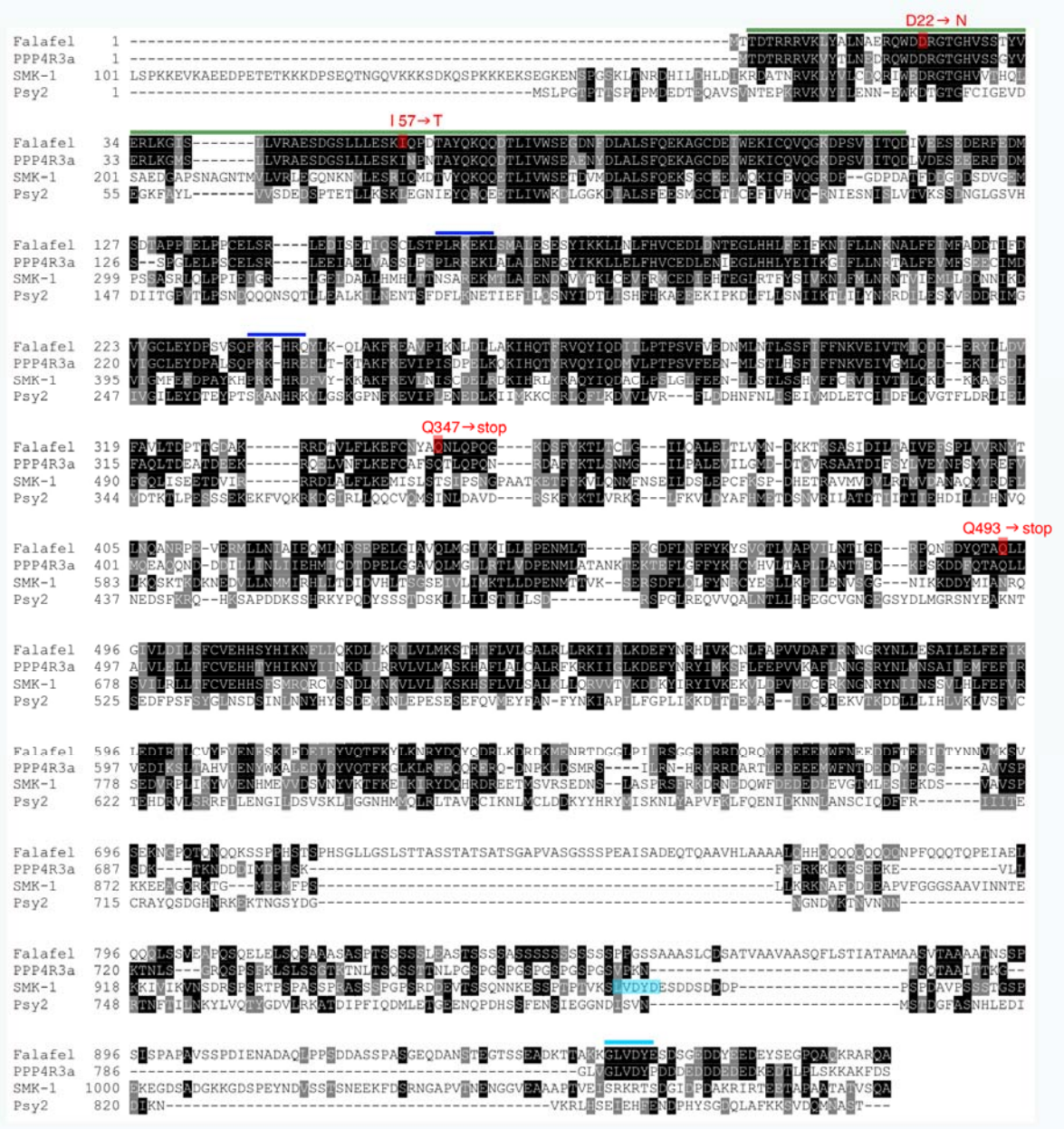
The EVH1 domain is found in multi-domain proteins implicated in a diverse range of signaling and nuclear transport events. Many EVH1-containing proteins also associate with actin-based structures and play a role in cytoskeletal organisation (Ball et al., 2002; Callebaut, 2002; Fedorov et al., 1999; Renfranz and Beckerle, 2002; Zettl and Way, 2002).

The amino acids 160-360 of Falafel constitute a domain of unknown function, DUF625. The domain architecture with an N-terminal RanBP domain followed by DUF625 is conserved in all proteins homologous to Falafel except the *C.elegans* protein, which has a predicted non-homologous sequence N-terminal to the EVH1 domain. Threading algorithms (Kelley et al., 2000) indicated that the middle part of Falafel (aa 120- 600, including the DUF625) as well as of the orthologous human proteins possesses a number of HEAT or armadillo repeats ((Gingras et al., 2005) and personal communication). The evolutionarily related ARM and HEAT motifs are tandemly repeated sequences of approximately 50 amino acid residues that form a conserved three-dimensional structure (Andrade et al., 2001). ARM/HEAT containing proteins function in various processes, including intracellular signaling and cytoskeletal regulation.

Additionally, two putative nuclear localization signals lie also at conserved positions (aa 158-164 and 236-241)

The Falafel C-terminal region of 300 amino acids rich in serines and glutamines is not conserved

except for a small box of 12 amino acids with a consensus LVDY at the extreme C-terminus (not aligned with the C.elegans LVDY motif, see turquoise box in Fig.12 A, not conserved in yeast Psy2). Interestingly, overexpression of a Falafel protein lacking this C-terminal part did not reproduce the full-length Falafel overexpression phenotypes, neither was it capable to rescue *falafel* mutants to adulthood (not shown). Therefore, despite its low conservation, this C-terminal part seems to be required for Falafel function.



A

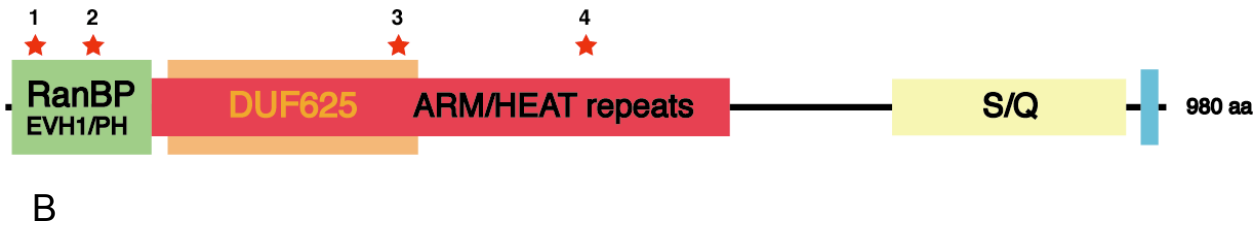


Figure 12. Falafel-like proteins are conserved throughout eukaryotae and share a similar domain architecture.

(A) Alignment of *Drosophila* Falafel with the orthologous proteins in human, *Caenorhabditis elegans* and budding yeast. Identities between any two proteins are in black and similarities are in grey. The green line marks the RanBP domain, dark blue lines indicate potential nuclear localization signals (NLS), and a putative conserved LVDY motif is colored in turquoise. The identified mutations (see Chap. 3.5.) are indicated with red color or, in (B), with numbered red asterisks, corresponding to the *flfl*^{1}, *flfl*^{2}, *flfl*^{3} and *flfl*^{4} alleles.

(B) Schematic view of Falafel. The green box indicates a domain with homology to the Ran binding domain of RanBP2 as well as moderate homology with pleckstrin homology (PH) and EVH1 domains. The orange box denotes a domain of unknown function, DUF625, present in all orthologs. The red box represents a number of armadillo (ARM)/HEAT repeats. A not conserved part that consists of many serines and glutamins is colored in yellow. The turquoise box at the extreme C-terminus indicates a novel conserved LVDY motif.

3.4. Falafel is a nuclear protein that is ubiquitously expressed

The expression of *falafel* was examined using RNA *in situ* hybridization. During embryogenesis *falafel* is expressed ubiquitously, possibly with a slight upregulation in the CNS (data not shown, comparable to the published expression patterns of the Berkeley *Drosophila* Genome Project). *falafel* is also ubiquitously expressed in larval tissues, e.g. in all imaginal discs, in the salivary gland, but not in the larval brain (data not shown).

The N-terminal RanBP domain in Falafel suggests a nuclear as well as a cytoplasmic localization of the protein. However, the predicted structural similarity of this N-terminal fragment of Falafel to the PH as well to the EVH1 domains would suggest further potential localizations to the membrane or cytoskeleton. Moreover, Falafel has two predicted nuclear localization signals (Fig. 12 A).

Falafel protein localization was followed by overexpressing an HA-tagged UAS-*falafel* construct. HA-antibody staining revealed an exclusive nuclear localization of Falafel (Fig.13).

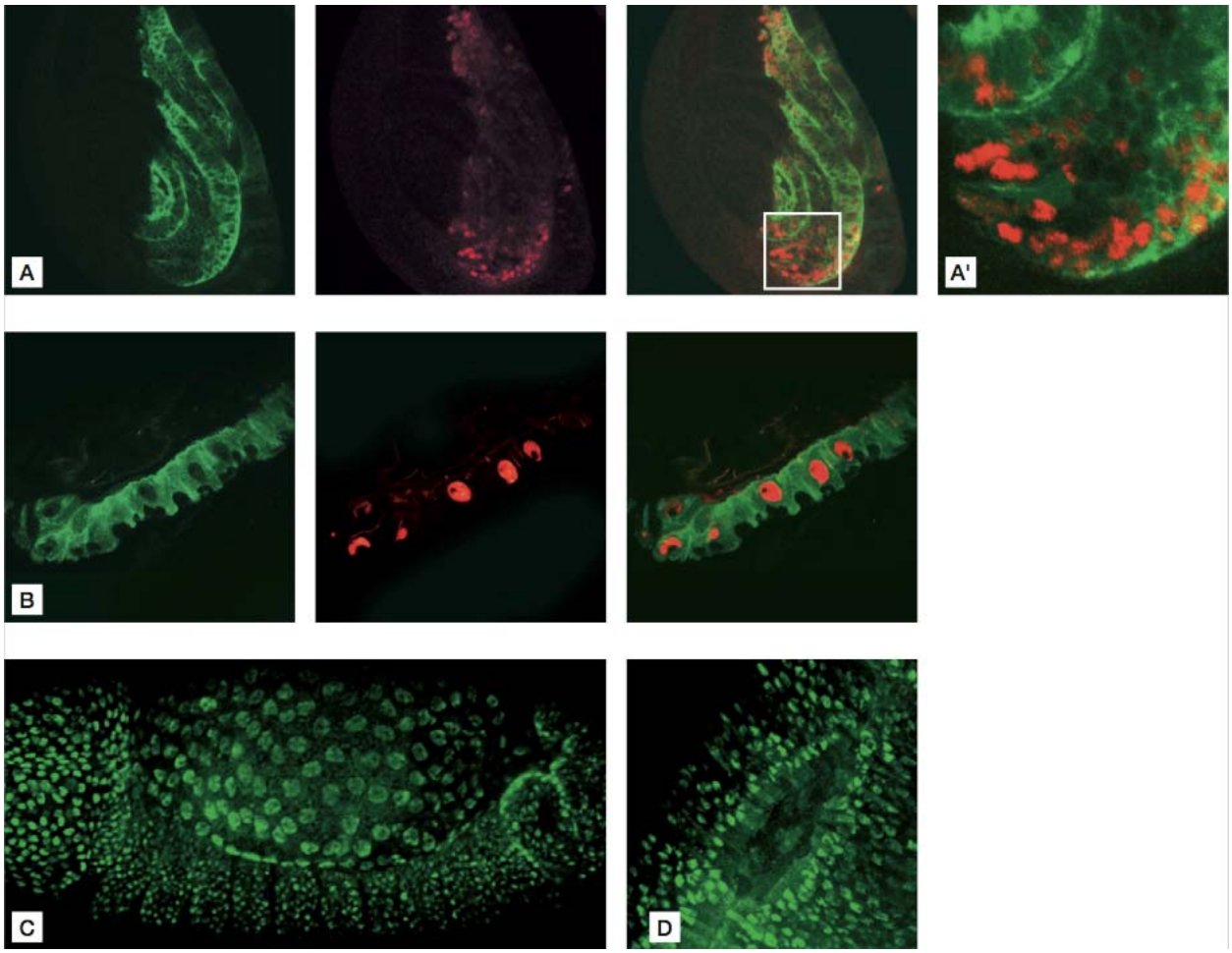


Figure 13. HA-tagged Falafel is located to the nucleus

Falafel protein localization was examined by using a transgene expressing hemagglutinin (HA) epitope-tagged Falafel (Falafel::HA) under the control of the UAS/Gal4 system. *HA-Flfl complemented a lethal heteroallelic flfl combination, demonstrating that it was fully functional.* Nuclear HA antibody fluorescence (shown in red in (A) and (B), and in green in (C) and (D)) is apparent in all cells throughout development. (A) Falafel::HA antibody staining in the nucleus and cytoskeletal GFP::actin fluorescence (shown in green) in leg imaginal disc cells (A) and gut cells (B) of wandering third instar larvae. UAS-falafel::HA and UAS-GFP::actin were expressed by using engrailed-Gal4.

(C) Falafel::HA antibody staining in a stage 13 embryo, at the onset of dorsal closure (lateral view), and (D) just before ultimate closure (dorsal view, detail). UAS-falafel::HA was expressed by using act5C-Gal4.

3.5. Generation of mutations in *falafel*

falafel was identified because of its overexpression phenotypes. In a rather artificial overexpression system the gene interacts with Drac1.

Double and triple mutants of the three known Racs in *Drosophila* display dorsal closure defects. Such defects are also caused by overexpression of either constitutive active or dominant negative Rac1 in the embryo. Overexpression of *falafel* does not seem to lead to severe embryonic phenotypes although some dead embryos with variable (also dorsal open) phenotypes have been observed (Fig. 11 F). In order to identify the potential role of Falafel during dorsal closure and related processes it is important to know the mutant phenotype. To generate mutations in *falafel* we made use of the phenotypes caused by the EP-dependent overexpression of the endogenous gene. EP47-082 produces a 100% penetrant wing phenotype with 69B-Gal4. In an EMS mutagenesis of EP carrying flies we screened 4000 flies for reversion of this overexpression phenotype to wild-type wings. In a second EMS mutagenesis we screened for reversion of pupal lethality caused by EP overexpression with actinGal4. In both screens revertants were recovered, five of them transmitted the mutation to the germ line (see materials and methods). Sequencing of four alleles identified mutations in *falafel* (Fig. 10 and 12). Two alleles, *flfl*^{1} and *flfl*^{2}, code for proteins with substitutions of well conserved amino acids in the RanBP1 domain. Interestingly, the *flfl*^{1} D22N mutation lies on a very conserved beta strand. The equivalent portion on RanBP is known to be involved in contacting the effector loop of Ran-GDP. The *flfl*^{2} mutation leads to a substitution of an isoleucine to a threonine at position 57 of the protein. The two other mutations in the *flfl*^{3} and *flfl*^{4} alleles give rise to truncated proteins. The *flfl*^{3} and *flfl*^{4} mutations are both substitutions of a glutamine to a stop codon at positions 347 and 493, respectively. Sequencing of the fifth revertant and thus potential *falafel* mutant (*flfl*^{5}) did not identify a mutation so far. Only the translated region of the gene was sequenced.

3.6. Mutant phenotype

flfl^{1} is homozygous viable, *flfl*^{2}, *flfl*^{3} and *flfl*^{4} are homozygous lethal. The deficiency Df(3)urd, which entirely deletes *flfl*, does not complement *flfl*^{3}, *flfl*^{4} and, at 29°C, *flfl*^{2}. However, Df(3)urd is viable in combination with *flfl*^{1} and *flfl*^{2} (at 25°C), indicating the presence of second hits on the *flfl*^{1} and *flfl*^{2} chromosomes reducing the viability. To confirm that loss of *flfl* is responsible for the lethal phenotype, we expressed a rescue construct containing UAS sequences driving a *flfl*

cDNA. Using the ubiquitous Gal4 driver line arm-Gal4, a few heteroallelic animals (*flfl*^{3}*/flfl*^{4}) are rescued to adulthood.

flfl^{3}*/flfl*^{4} and *flfl*^{3or4}*/Df(3)urd* heteroallelic mutant embryos display a variable and not fully penetrant dorsal closure and head involution defect (Fig. 14 A and B).

The phenotype reminded a hungry scientist of a (“dorsally”) opened-up bread that is filled with salad and small so-called falafel balls composed of chick-peas, hence the name. Only 20% of the presumptive *flfl*^{3}*/flfl*^{4} and *flfl*^{3or4}/Df(3)urd heteroallelic mutant embryos display dorsal closure and/or head involution defects. The remaining mutant embryos develop further and die during larval stages (until late 3rd instar).

The low penetrance of the dorsal open phenotype could be due to the endurance of maternal gene product. Maternal Falafel activity was eliminated by generating *falafel* homozygous germ line clones (GLCs, Chou and Perrimon 1996). *flfl*^{2}*,* *flfl*^{3} and *flfl*^{4} GLCs produced very few embryos, some of which displayed dorsal closure or germ band retraction defects (Fig. 14 C and D). This variable phenotype of homoallelic *flfl* mutants could possibly be due to second hit mutations. No embryonic defective phenotypes have been observed for *flfl*^{1} GLCs.

Animals possessing the heteroallelic combinations of *flfl*^{1} or *flfl*^{2} with *flfl*^{3}*,* *flfl*^{4} or Df(3)urd reached adulthood and displayed a statistically significant reduction of body size and weight (Fig. 14 E and F). The measured weight reduction lied between 10 and 30% as compared with their wild-type counterparts, depending on the allelic combination used. The size of single ommatidia seemed to be normal. This suggests that the reduction of overall body size is due to decreased cell number.

The fact that body size in hemizygous hypomorphic *falafel* flies (e.g., *flfl*^{1}/ Df(3)urd) is not more reduced as compared to flies with heteroallelic *falafel* combinations (e.g., *flfl*^{1}*/flfl*^{3}) suggests that *flfl*^{3} (and *flfl*^{4}) are strong – possibly null – alleles, at least with respect to growth. Furthermore, the *flfl*^{3} and *flfl*^{4} alleles potentially give rise to truncated proteins in which the last 633 (*flfl*^{3}) or 487 (*flfl*^{4}) amino acids are missing. Overexpression of a *flfl* transgene in which 300 amino acids of the C-terminus were truncated did not reproduce the full-length Falafel overexpression phenotypes (not shown). This finding additionally supports the notion that *flfl*^{3} and *flfl*^{4} are strong alleles.

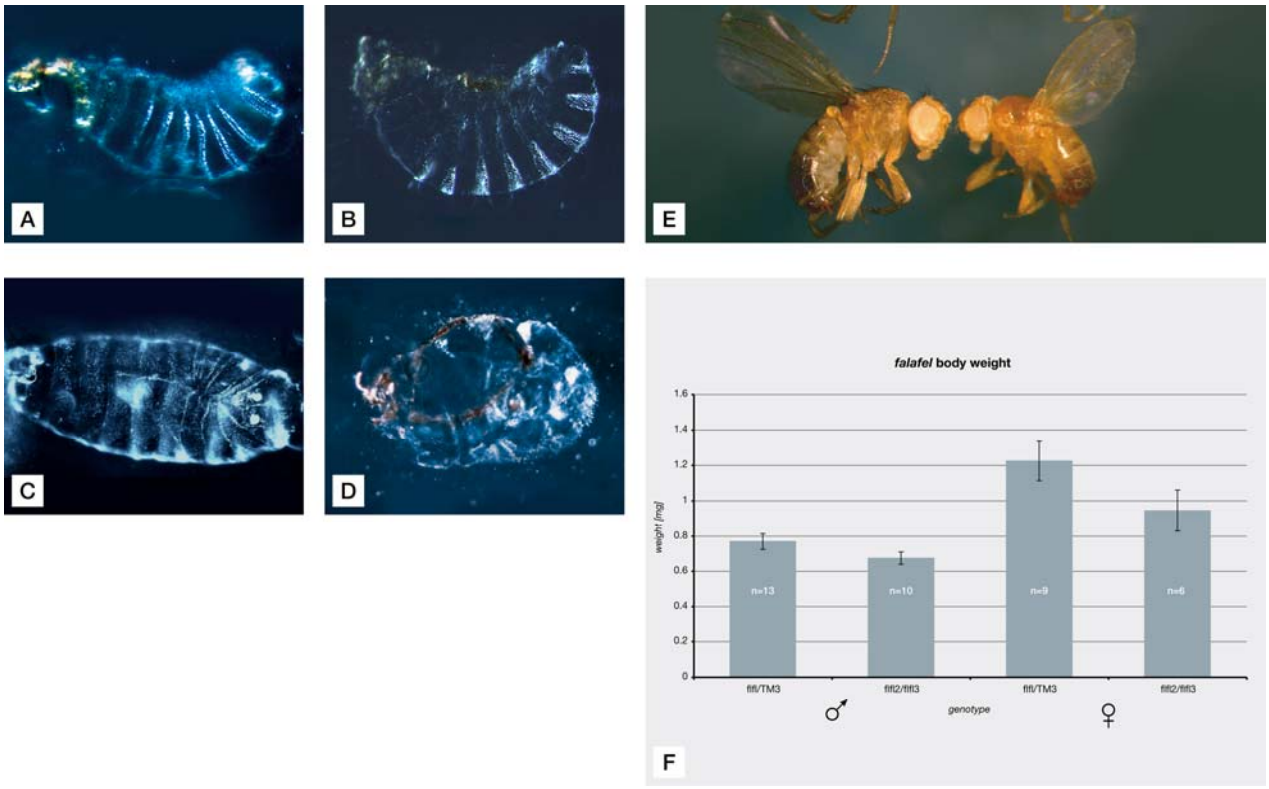


Figure 14. *falafel* mutant phentoytpe

(A-D) Cuticle preparations of stage 13 embryos, lateral views, with anterior at left. *flfl*^{3}/ Df(3)urd (A) and *flfl*^{4}/ Df(3)urd (B) embryos display dorsal closure and head involution defects.

(C, D) germ line clones. (C) *flfl*^{5} germ line clone embryo with slight puckering of dorsal midline due to imperfect dorsal closure. (D) *flfl*^{2} germ line clone embryo with large dorsal/anterior hole. *flfl*^{3}, *flfl*^{4} germ line clones produced very few embryos, some of which displayed dorsal closure or germ band retraction defects.

(E, F) Body size and weight reduction of *falafel* mutant flies with viable heteroallelic combinations. (E) *flfl*^{2}/*flfl*^{3} mutant fly (right) is reduced in size compared to the control (y, w; TM3 balancer, y^{+} marked, left). (F) *flfl*^{2}/*flfl*^{3} mutant flies display a statistically significant reduction of weight of 12% (males) and 23% (females) as compared to controls (y, w; TM3 balancer).

3.7. *falafel* orthologs in other organisms

3.7.1. RNAi against the *C. elegans falafel* orthologue SMK-1

The *C. elegans* genome encodes a single ortholog of *falafel* (*smk-1*). We addressed the question whether this gene would have similar functions in cell migration processes. For example, it would be interesting to know whether *smk-1* loss-of-function affects ventral enclosure in *C.elegans*, a process similar to dorsal closure in *Drosophila*. Interestingly, *smk-1* RNA

interference resulted in embryonic lethality with a high penetrance. Most of the embryos started to develop normally, but displayed several general defects in morphogenesis late in embryogenesis (Fig. 8). However, ventral enclosure did not seem to be exclusively affected.

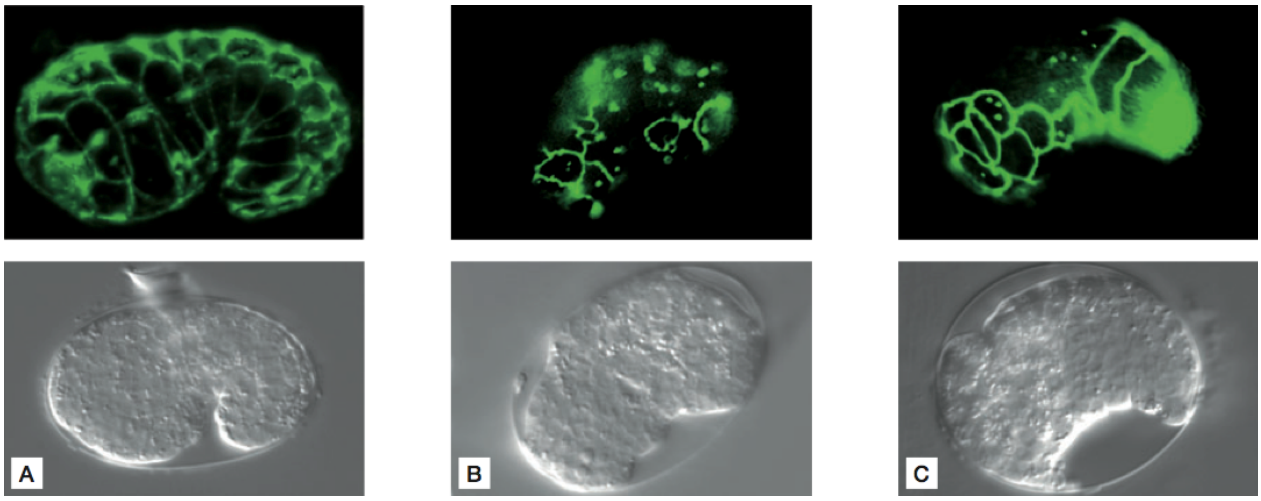


Figure 14. RNAi phenotype of the Caenorhabditis elegans *falafel* ortholog

(A, upper panel) Wildtype worm embryo expressing the adherens junction marker JAM::GFP, below the corresponding DIC image. (B,C) F1 embryos after RNAi treatment. SMK-1 RNAi leads to lethality during embryonic development with a high penetrance. Dead embryos display various morphogenesis defects.

Interestingly, a recent report revealed SMK-1 as a stress responsive protein that regulates the FOXO transcription factor DAF-16 (Wolff et al., 2006). Furthermore, the authors showed that a *daf-2* (Insulin receptor) mutant mediated longevity phenotype was completely suppressed by *smk-1* RNAi. Like Falafel, SMK-1 also localized predominantly to the nucleus.

Thus, the interaction of *smk-1* with components of the insulin pathway possibly provides an explanation for the reduced body size phenotype observed in *falafel* mutants. Moreover, the fact that SMK-1 is required for DAF-16 mediated transcription of oxidative stress responsive genes (Wolff et al., 2006) fits well with the herein proposed function of Drac1 and some of the identified suppressors in oxidative stress response.

3.7.2. The yeast and human orthologues of Falafel function in a protein phosphatase 4 (PPP4) complex

There is one yeast ortholog of *falafel*, PSY2 (Fig. 12 A). Two independent studies analyzed protein complexes in large scales and identified PSY2 in a phosphatase complex, composed of Pph3 and YBL046w, the putative orthologs of the PP2A-like PPP4 catalytic and regulatory subunit 2, respectively (Gavin et al., 2002; Gavin and Superti-Furga, 2003; Ho et al., 2002). A recent report (Keogh et al., 2006) identified the same complex by tandem-affinity-purification (TAP) tag purification (Rigaut et al., 1999). In addition, a two-hybrid interaction was reported for PSY2 and YBL046w, which indicates a probable direct interaction (Gingras et al., 2005; Ito et al., 2001).

There are two highly related human Falafel orthologs PPP4R3α and β (KIAA2010 and KIAA1387, respectively). Using TAP tags, it has been shown that PPP4R3α and β co-purified specifically with the catalytic subunit of the serine/threonine phosphatase PPP4 (PPP4c) and the regulatory subunit, PPP4R2 (Gingras et al., 2005) (see supplementary paper).

Preliminary results of pull-down experiments indicate that the portion of Falafel able to assemble with PPP4R2/PPP4C is located between aa 213-719, corresponding to the DUF-625 domain and the predicted ARM/HEAT repeats (A.C. Gingras, personal communication). Thus the mutations that were obtained in the N-terminal RanBP domain of falafel (*flfl*^{1} *and flfi*^{2}) are probably not affecting the binding to PPP4C/PPP4R2. The two stop mutations (*flfl*^{3} and *flfl*^{4}*)* at aa positions 347 and 497, however, give rise to truncated proteins that probably display an impaired or abolished binding to the phosphatase complex components.

3.7.3. Cisplatin sensitivity of Falafel and the yeast and human PPP4 complex components: Function in DNA damage response?

It was shown that deletion of the three components of the yeast PPP4 complex, PPH3, YBL046w and PSY2 rendered cells hypersensitive to cisplatin treatment (Gingras et al., 2005; Hastie et al., 2006; Wu et al., 2004). Importantly, the hypersensitivity of psy2 deletion could be reverted by expression of the human ortholog PP4R3, indicating that PSY2 and PPP4R3 are functionally equivalent in mediating resistance to cisplatin (Gingras et al., 2005). Furthermore, we showed that loss of *falafel* function also leads to hypersensitivity to cisplatin in flies (Gingras et al., 2005) (see supplemented paper).

Cisplatin is a DNA-damaging agent that is used as a chemotherapy drug to treat various types of cancers. It acts by crosslinking DNA in several different ways. Normally, damaged DNA sets off DNA repair mechanisms, which can also activate apoptosis when repair proves impossible. Conversely, defective repair mechanisms can lead to accumulations of mutations and eventually to cancer. Therefore, the hypersensitivity to cisplatin suggests for the PPP4 complex a function

in the regulation of DNA repair.

Through progression of the cell cycle, several factors at specific checkpoints control if extensive DNA damage has occurred or if a key event, such as the attachment of a chromosome to the mitotic spindle, has not occurred properly. These checkpoints help to ensure that a cell divides only when it has completed all of the molecular prerequisites for producing healthy daughter cells.

Intriguingly, an important regulator of DNA damage checkpoint response, RAD53 (Chk2, *loki* in flies) was also reported to interact with PSY2 in yeast two-hybrid assays (Gingras et al., 2005; Uetz et al., 2000). Chk2 is a cyclin-dependent kinase that drives cells into mitosis. When DNA is damaged in response to genotoxic insults, Chk2 plays an essential role in inducing a cell cycle arrest through the ATM/Chk2 pathway in *Drosophila* (Masrouha et al., 2003). In the absence of *Drosophila Chk2/loki* function, there is failure of chromosome segregation probably due to damaged or incomplete replicated DNA. Interestingly, this phenotype was partially suppressed by reducing the gene dosage of *cdc25/string* (Xu and Du, 2003). Human Cdc25A is a known target of Chk2, which itself gets activated by ATM in response to DNA damage (reviewed by (Busino et al., 2004). *Drosophila cdc25, string,* was also identified in this work as a suppressor (see Chap. 2.4. and discussion Chap. 2.9.4.). *string* also dephosphorylates Cdc2 and Cdk2, positive regulators of cell division. The cyclin-dependent kinase Cdc2 normally drives cells into mitosis, and it is the ultimate target of pathways that mediate rapid arrest in G2 in response to DNA damage (reviewed by (Stark and Taylor, 2006). We tested *Drosophila cdc2* for genetic interaction with *falafel.* Preliminary results indicated indeed a dominant enhancement of body size reduction of viable *flfl* allelic combinations when *cdc2* function is impaired (in $cdc2^{E1\text{-}23}$ heterozygous mutants, data not shown).

Another connection between the PP4 complex and DNA damage is demonstrated by the fact that the yeast PP4 complex dephosphorylates the histone H2 variant H2A (Keogh et al., 2006). H2A (H2AX variant in higher eukaryotes) is phosphorylated by ATM, ATR and DNA-PK in response to DNA double-strand-breaks (DSB) (Rogakou et al., 1998; Stiff et al., 2004) (reviewed by (Fernandez-Capetillo et al., 2004). This modification is important for recruiting numerous DSB-recognition and repair factors to the break site, including DNA damage checkpoint proteins. Keogh et al. showed that the yeast H2AX dephosphorylation by PPP4 is necessary for efficient recovery from the DNA damage checkpoint, thereby allowing cells to enter the cell cycle again. All three components of the yeast PPP4 complex, Pph3, Ybl046w and Psy2, were also isolated in TAP tag affinity purifications with the H2A and H2B subtypes, HTA1, HTA2, HTB1 and HTB2 (Krogan et al., 2006). All these histones have been shown to have a role in the DNA damage response or in DSB formation during meiosis.

Interestingly, one of the human PPP4 complex components, PPP4R2, physically interacts with histone H1 (A.C. Gingras, personal communication). It can be speculated that through this interaction the phosphatase complex is brought in proximity to the histone H2 substrate.
Several reports suggest that ATM activation and H2AX phosphorylation are also induced by ROS-mediated DNA damage (reviewed by (Tanaka et al., 2006). Thus it is conceivable that the PPP4 complex and the herein identified subunit Falafel/PPP4R3 are involved in ROS induced DNA damage response. As proposed in Chapter 2.9.2., four Dcdc42^{N17} and two Drac1^{N17} suppressors are possibly also involved in some aspects of ROS induced signaling.
Additionally, the catalytic and the regulatory subunit R1 of PPP4 were co-purified with the histone deacetylase HDAC3 and PPP4 was shown to negatively regulate HDAC3 (Zhang et al., 2005), demonstrating a further link between PPP4 and histone regulation. In this context it is interesting to note that HDAC3 has been shown to suppress the transcriptional activity of c-Jun (Weiss et al., 2003). This HDAC3-dependent suppression was relieved by phosphorylation by JNK.

Further links between the PPP4 complex and DNA damage were described and are discussed in the supplementary paper (Gingras et al., 2005).

3.7.4. PPP4, JNK signaling and stress

JNK was recently also shown to phosphorylate H2AX in response to UV stress (Lu et al., 2006). The Drosphila PPP4 subunit Falafel was originally identified as a Drac1 interactor, thus the finding that JNK as well as the yeast PPP4 complex regulate H2AX phosphorylation state is particularly interesting since Drac1 has been shown to activate JNK in several systems.
Furthermore, human PPP4R3 (Falafel ortholog) was reported to be phosphorylated in response to stress (referred to as unpublished data in (Wolff et al., 2006)). Moreover, PPP4 was shown to positively regulate JNK in response to TNF-α signaling (Zhou et al., 2002). The requirement of PPP4 for JNK and AP-1 activity was also shown by Inostroza et al. (2005) (Inostroza et al., 2005). In addition, PPP4 acts as a positive regulator of hematopoietic progenitor kinase 1 (HPK1), a Ste20-like protein kinase acting upstream of JNK (Zhou et al., 2004). Interestingly, the *Dictyostelium discoideum* Falafel ortholog SMEK was identified as a suppressor of MEK1, a member of the MAPKK family of kinases (Mendoza et al., 2005). SMEK suppresses MEK1 null cell polarity, chemotaxis, and gene expression defects. In addition, SMEK was shown to be required for proper cytokinesis and myosin II assembly.

In *C.elegans*, JNK also directly interacts with and phosphorylates the FOXO transcription factor DAF-16 (Oh et al., 2005), which is interesting since the Falafel ortholog SMK-1 was shown to be required for several (including oxidative) stress responsive functions of DAF-16, and genetic analysis revealed an interaction with the insulin/IGF-1 receptor DAF-2 (Wolff et al., 2006).
A role of PPP4 in insulin signaling is further supported by the fact that degradation of the insulin receptor substrate 4 protein (IRS-4) stimulated by TNF-α is dependent on PPP4 (Mihindukulasuriya et al., 2004). Moreover, okadaic acid, a potent PPP4 inhibitor, was shown to increase IRS-4 phosphorylation induced by Insulin (Villarreal et al., 2006).

3.8. PPP4 and the centrosome

In *Drosophila*, orthologous proteins for the catalytic subunit and the second regulatory subunit of the PPP4 complex exist, Pp4-19C and PPP4R2, respectively. The two proteins have been isolated in one complex (Hastie et al., 2000), and recently Falafel has also been shown to be part of this complex (Hastie et al., 2006). As PPP4c in human cells (Brewis et al., 1993), Pp4-19C localizes to centrosomes and seems to be an essential protein for nucleation, growth, and stabilization of microtubules at centrosomes during cell division (Helps et al., 1998). There are two PPP4c orthologous proteins in *C.elegans*, PPH-4.1 and PPH-4.2. As PP4-19C in *Drosophila*, PPH-4.1 also localizes to centrosomes and is required for the maturation and microtubule nucleation of the centrosome (Sumiyoshi et al., 2002). Furthermore, PPH-4.1 appears to play a role in either the establishment or the maintenance of chiasmata between homologous chromosomes during meiosis.
Interestingly, in mitotic cells, activated ATM was also shown to be localized at centrosomes (Oricchio et al., 2006). The authors suggested that ATM could thereby monitor spindle integrity during mitosis. Thus, it seems that both PP4 and ATM are not only involved in a DNA damage response but also in further cell cycle defect responses, perhaps in the same pathway?

3.9. PPP4 and Rac?

falafel was identified as a suppressor of a Drac1^{N17}-mediated rough eye phenotype. What could be the physiological relevance of this interaction? So far, no interaction between Rac and the PPP4 complex has been reported. PP2Ac, a phosphatase similar to PPP4, seems to negatively regulate the actin cytoskeleton via a TAP42 and Rho GTPase-dependent mechanism (Wang and Jiang, 2003). TAP42 was shown to interact with all PP2a-like phosphatases, including PPP4 (Chen et al., 1998; Gingras et al., 2005). One might speculate that TAP42 and PPP4 could be

involved in a similar Rho or Rac GTPase signaling pathway. It will be interesting to know whether the small GTPase Ran binding protein domain (RanBP) in Falafel could bind the small GTPase Drac1. So far, no interaction could be detected between Falafel with either Ran or Rac by co-immunoprecipitation assays ((Gingras et al., 2005) and personal communication)). Intriguingly, *Drosophila* RhoGAP68F has been shown to interact with PP4-19C (PPP4c) in a yeast two-hybrid assay (Stanyon et al., 2004). Thus, Falafel and the PPP4 complex might regulate Drac1 through RhoGAP68F. Using affinity purification and mass spectrometry, the yeast Falafel orthologous protein Psy2 was also found in complex with two RhoGAPs, BEM2 and RGA2 (Gavin et al., 2006; Gavin et al., 2002), further indicating a link between the PPP4 complex and Rho GTPases. We have identified two mutations that lead to substitutions of conserved amino acids in the RanBP domain of Falafel (*flfl*^{1} and *flfl*^{2}). These mutant alleles could be used to investigate possible genetic interactions with RhoGAP68F or other RhoGAPs.

3.10. Open questions

There are now some good clues about the function of Falafel. In summary, they suggest that Falafel functions as a regulatory subunit of the PPP4 complex that is involved in DNA damage repair in response to a number of stresses. However, several questions remain to be addressed, especially concerning the body size and dorsal closure phenotypes of *falafel* mutants and the connection to Drac1 signaling.

With respect to the reduced body size of viable *falafel* allelic combinations it will be worthwhile to examine possible genetic interactions with components of the insulin pathway, which has a leading role in growth control. As mentioned above, the Falafel *C.elegans* ortholog SMK-1 genetically interacts with DAF-2, the *C.elegans* Insulin receptor. Since *smk-1* RNAi completely suppresses the *daf-2* mutant longevity phenotype it will be interesting to see whether *falafel* mutants display a longevity phenotype. The fact that the *daf-2/InR* mutant phenotype is suppressed by *smk-1* RNAi suggests that SMK-1 and PPP4 act as a negative regulator of Insulin signaling. On the other hand however, the *falafel* hypomorphic mutant phenotype – reduced body size – would be contradictory to a negative role in Insulin signaling.

Interestingly, small interfering RNAs directed against the human *falafel* ortholog PPP4R3β shorten mitotic transit time (Kittler et al., 2004). A shortened cell division cycle eventually leads to smaller cells since they have less time to grow. Additionally, RNA interference against *Drosophila* PPP4c (PP4-19C) caused a 20% reduction in growth of *Drosophila* Schneider cells (Silverstein et al., 2002). I did not observe a clear reduction of cell size at the level of ommatidial size. Since the overall body size reduction was about 10-30% it is possible that an ommatidial

size reduction in a similar range was overseen. The cell number was not examined.

flfl^{3} and *flfl*^{4} heteroallelic embryos display a dorsal closure defective phenotype. This is consistent with the role of Drac1 in dorsal closure. The reported interactions of the PPP4 complex with the JNK pathway (see above) further indicate that Falafel could act in a Drac1/JNK signaling pathway. A hypomorphic PP4-19C mutant does not show dorsal closure defects (Helps et al., 1998). Additional loss-of-function alleles of PP4-19C and the second regulatory PPP4 subunit (R2) will possibly elucidate the potential role of PPP4 in dorsal closure. Interestingly, all eight subunits of the TRiC/CCT complex were co-purified with PPP4c (Gingras et al., 2005) as well as with TAP42 (Ho et al., 2002). The TRiC/CCT complex is an ATP-dependent chaperonin that mediates protein folding in the eukaryotic cytosol. Genetic and biochemical data initially showed that it is required for the folding of the cytoskeletal proteins actin and tubulin, but a number of recent reports described other proteins that require TRiC/CCT for proper folding (Dunn et al., 2001). The involvement of PPP4 interactor TAP42 in actin regulation was already mentioned (see above). Therefore, one might speculate that the interactions between actin/tubulin, TAP42, TRiC/CCT and PPP4 could contribute to either remodeling of the cytoskeleton that occurs during dorsal closure as well to microtubule nucleation at centrosomes.

A further indication for the relevance of the dorsal closure defective phenotype of *falafel* mutants and of a putative role of PPP4 in the regulation of the cytoskeleton stems from studies with the *Dictyostelium discoideum* Falafel ortholog SMEK. SMEK is required for cell polarity, proper cytokinesis and myosin II assembly (Mendoza et al., 2005).

4. Conclusion

The initial aim of this study was to identify novel signaling components that act during dorsal closure. Therefore known key players in dorsal closure, Drac1 and Dcdc42, were used as starting points for a genetic screen. Dominant negative Drac1 or Dcdc42 (N17) were mis-expressed in the eye, the resulting rough eye phenotype was suppressed by co-overexpression of several EP-lines as described. This screening system is rather artificial but it is validated by the fact that the mutations generated in one of these suppressor lines (*falafel*) cause dorsal open phenotypes. In addition, new functions for Rac and Cdc42 signaling in body size control, ROS homeostasis, and DNA damage repair could possibly have been revealed. Another argument for this kind of gain-of-function screen is that genes can be identified that possibly do not have any loss-of-function phenotype, or that have, like *falafel*, no clear phenotype that would be identified in loss-of-function screens.

4.1. Falafel

falafel overexpression in two EP lines was identified to suppress the $Drac1^{N17}$-mediated rough eye phenotype. *falafel* overexpression leads to further phenotypes including a thorax closure defect in adult flies. Thorax closure resembles dorsal closure in both mechanistic and molecular ways. HA-tagged Falafel was shown to localize predominantly to the nucleus and RNA *in situ* hybridization revealed a ubiquitous expression of *falafel* transcripts. EMS-generated mutations in *falafel* were easily identified as reversions of two specific overexpression phenotypes. The mutant phenotype included a not fully penetrant dorsal closure defect and a reduced body size of the viable allelic combinations.

Furthermore, the RNA interference phenotype of the *C.elegans falafel* ortholog *smk-1* was investigated. The animals displayed several morphogenesis defects late in embryogenesis. Ventral enclosure, a process similar to dorsal closure, did not seem to be exclusively affected.

Several reports in yeast, human cells and recently also in *Drosophila* eventually showed Falafel and the orthologous proteins to be part of the tripartite protein phosphatase 4 (PPP4) complex. Furthermore, components of this complex were shown in all three systems to be sensitive to cisplatin indicating that Falafel and PPP4 could be involved in DNA damage repair. This is further supported by the reported interactions of yeast Falafel (Psy2) with the cell cycle checkpoint protein Rad53 (Chk2) as well as of yeast and mammalian PPP4c with H2AX (see above).

Cisplatin is commonly used as an anti-cancer agent. Resistance of cancer cells to cisplatin is a major impediment to the clinical success of this drug. The cisplatin sensitivity of *falafel* mutant flies offers therefore a good oppurtunity to get a better understanding of the signaling pathways involved, potentially leading to the design of new anti-cancer drugs. It will be interesting to know whether mutations in one of the three *Drosophila* Rac genes possibly give rise to cisplatin sensitive flies as well.

A further function of the PPP4 complex in stress response mediated by the JNK and the insulin/FOXO pathways is suggested by a number of reports. This is intriguing, since Falafel, as a part of the PPP4 complex, was identified as a suppressor of a Drac1 dominant negative ($Drac1^{N17}$) phenotype. Falafel is therefore a putative positive regulator of Drac1 signaling, which itself has also been shown to activate the JNK pathway, possibly through Falafel and PPP4. This is additionally supported by the reported interactions of RhoGAPs with components of the PPP4 complex (see above).

In *Drosophila*, as well as in human and *C.elegans*, components of the PPP4 complex localize to mitotic centrosomes. Interestingly, ATM, a candidate upstream regulator of PPP4 (see above), also localizes to centrosomes, possibly as a checkpoint regulator to control the integrity of the spindle apparatus before allowing cells to progress further through the cell cycle. This suggests that both PPP4 and ATM are not only involved in a DNA damage response but also in responses to other defects that can occur during cell division.

4.2. EP screen suppressors: Potential role in the regulation of ROS, DNA damage response and cell cycle

The rough eye phenotype of the Drac1/Dcdc42 tester flies was specifically suppressed by 23 EP element insertions that correpond to 17 genes, none of which has been previously reported to interact with Drac1 or Dcdc42. The identified genes represent a rather heterogeneous group whose products are involved in a variety of cell signaling, transcriptional regulation, cytoskeletal and other events. The function, if known, of each of these genes and their potential role in Rho GTPase signaling and dorsal closure are discussed in chapter 2.9.

Initially, for three of these genes (*SelD, dTrf2* and *dATP7A*) it was apparent that they probably interfere with cellular ROS (reactive oxygen species) levels. After a further evaluation of all identified suppressor genes I hypothesized a potential role in ROS regulation for three additional genes (CG30421, CG6700/*NOS* and *pfrx,* see chapter 2.9.2.).

ROS have been traditionally regarded as toxic by-products of metabolism. Furthermore, ROS have been implicated in several human processes and diseases, including cancer, heart disease, and neurodegenerative diseases. Because of their high reactivity, ROS bring about damages to various cellular macromolecules, including DNA. The identification of *falafel*, which is possibly involved in a DNA damage response, as a suppressor of $Drac1^{N17}$ could therefore be in line with the other genes proposed to have a function in ROS signaling. In order to counteract oxidative DNA damage, cells have evolved a wide variety of adaptive cellular responses ranging from transient growth arrest to permanent growth arrest, apoptosis or necrosis. Importantly, the JNK pathway has been demonstrated to play essential roles in the cellular protective response against ROS and other forms of stress. This is of particular interest since JNK has been shown to be regulated by small Rho GTPases.

Accumulating evidence implicates ROS also in normal physiological signaling by growth factors and cytokines. The primary sources of ROS that are involved in such signaling pathways are plasma membrane oxidases, preferentially NADPH oxidases. Cdc42 and especially Rac1 and Rac2 have well-documented roles in the regulation of NADPH oxidases. This suggests that the rough eye phenotype of the $Drac1^{N17}$ and $Dcdc42^{N17}$ tester flies could be due to oxidative damage or misregulation of cellular redox states in the affected cells. Therefore, one possible mechanism for the suppression phenotype is a direct regulation of ROS levels by the responsible genes. Interestingly, endogenously produced ROS (via Rac stimulated NADPH oxidases) have been demonstrated to be important mediators of actin cytoskeleton remodeling (Irani and Goldschmidt-Clermont, 1998 and Moldovan et al., 1999). Thus, the rough eye phenotype could be due to a misregulation of the cytoskeleton. Further experiments with Drac and Dcdc42 mutants and with mutants of the identified suppressor genes to test the potential hypersensitivity to oxidants remain to be performed.

Furthermore, several studies have demonstrated that ROS may also play an important role in cell cycle progression. Interestingly, ROS have been shown to cause a cell cycle arrest at the G2/M transition (Thorn et al., 2001; Chung et al., 2002; Zhang et al., 2001; Zhang et al., 2003 and Bijur et al., 1999). It is thus conceivable that the identified suppressor genes could interfere with cell proliferation, possibly via regulation of ROS. Importantly, two known regulators of cell proliferation, *Rbf* and *string* (*Drosophila* cdc25), were identified as suppressors of $Dcdc42^{N17}$, as well as the gene encoding the transcription factor dTrf2. Together with DREF, dTrf2 has been demonstrated to regulate transcription of genes that are involved in cell proliferation, DNA replication and DNA damage protection. DREF binds to the promoter-activating element DRE (DNA replication-related element). Interestingly, two such DRE sequences lie in the 5' region of the gene encoding the catalytic subunit of the PPP4 complex in *Drosophila*, *PP4-19C*. Moreover,

the yeast Falafel ortholog Psy2 has been shown to interact with the important cell cycle checkpoint regulator Rad53/Chk2, and preliminary results in *Drosophila* indicate that *falafel* and *cdc2* interact genetically. In addition, the Drac1^{N17} suppressor SelD, which is implicated in the response to oxidative stress, may also be involved in cell cycle regulation (Alsina et al., 1999; Alsina et al., 1998; Serras et al., 2001).

In summary, of the 17 genes that have been identified in the EP screen nine are possibly involved in ROS signaling, DNA damage response or cell cycle regulation. These three processes are surely linked together: ROS can be expected to trigger cell cycle checkpoint responses directly via the induced damage to DNA or indirectly via various signaling pathways. Thus, these nine genes possibly represent a group of genes whose products take part in a Dcdc42 or Drac1 mediated response to stress and subsequent regulations of the cell cycle (schematically summarized in Fig.16). The nine genes are: *dTrf2, dATP7A, CG6700 (NOS?), CG30421, Rbf* and *stg* (Dcdc42^{N17} suppressors), *pfrx, SelD* and *flfl* (Drac1^{N17} suppressors). Except for *dATP7A* and *stg*, none of them was implicated in Dcdc42/Drac1 signaling before. On the other hand, the role of Cdc42 in cell cycle regulation and of Rac1/2 (and to a lesser extent of Cdc42) in the regulation of NADPH oxidase and thereby of ROS has already been appreciated. The identification of the nine genes as suppressors of Dcdc42^{N17} or Drac1^{N17} additionally supports a role of the small Rho GTPases in the regulation of ROS and of the cell cycle. Further analysis of these nine genes could also shed light on new modes of ROS and cell cycle regulation.

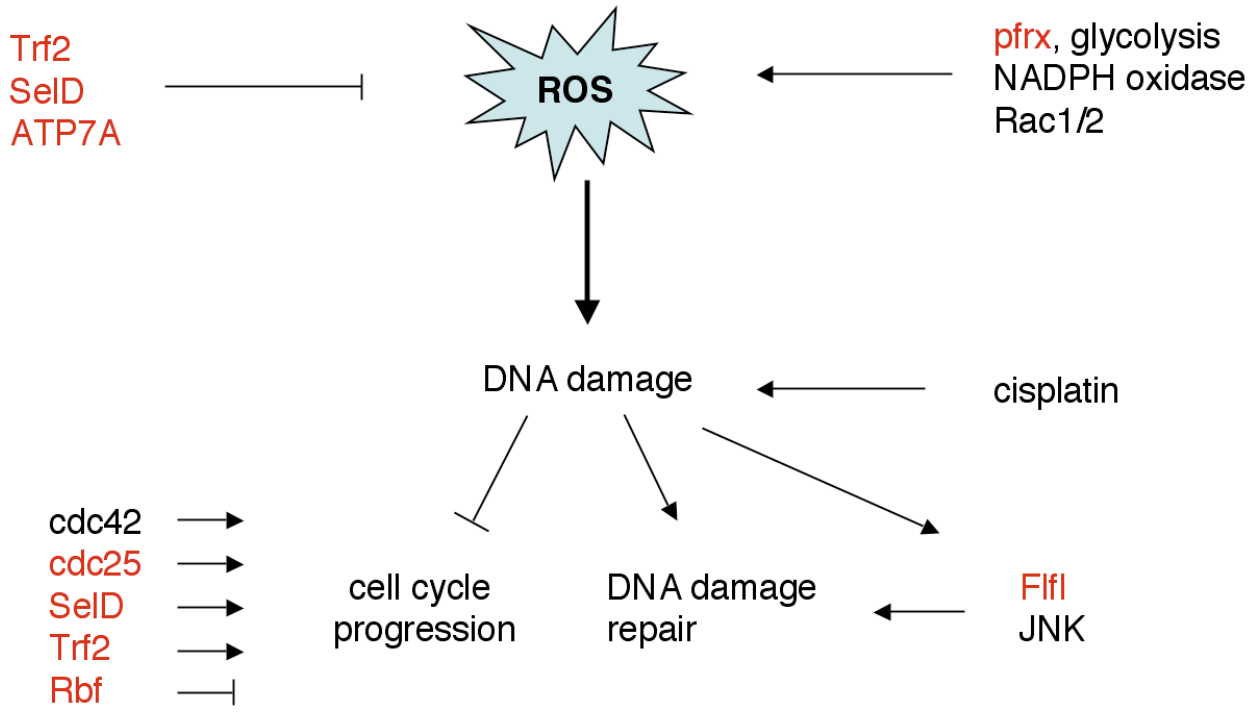


Figure 16. Model of ROS signaling, DNA damage response and cell cycle progression in the context of some of the herein identified Drac1/Dcdc42 suppressor genes

Oxidative stress by reactive oxygen species (ROS) induces both damage to DNA as well as signaling pathways leading to cell cycle arrest and DNA damage repair. Rac1/2 (and possibly Cdc42) regulate ROS levels through the NADPH-oxidase enzyme complex. Cdc42 positively regulates cell cycle progression (G2/M transition). The identified suppressors with a proposed function in ROS signaling, DNA damage response and cell cycle progression are shown in red.

Eight further genes were identified as Dcdc42^{N17} or Drac1^{N17} suppressors. These genes encode transcription factors, RNA and DNA binding proteins and cytoskeleton proteins. Their exact roles in Drac1 or Dcdc42 signaling remain to be assessed.

To conclude, our genetic approach of systematic gene overexpression successfully identified novel interactors of Drac1 and Dcdc42. Several novel interactions suggest additional functions for Drac1 and Dcdc42 in ROS signaling, DNA damage response and cell cycle progression. Furthermore, in the case of one interactor, *falafel*, we could show that a screen in the fly eye can be used as a valid assay for targeting genes that function during dorsal closure.

5. Material and Methods

Fly stocks

Flies were grown at 25°C on standard food. It contains the following ingredients per 1 liter: 75g glucose/dextrose, 55g maize, 10g flour, 8g agar, 15ml nipagin (33g/l EtOH)/nipasol (66g/l EtOH), and 100g live yeast. Crosses were performed at 25°C except for crosses with GMRGal4>Drac1^{N17} and Sev>Dcdc42^{N17}, which were performed at 18°C. Most fly stocks have been described previously:

GMR-Gal4 (Freeman, 1996)

sev-Gal4 (sevE-hsP-Gal4 K25, generated in our lab)

UAS-Drac1^{N17} (Luo et al., 1994))

UAS-Dcdc42^{N17} (Luo et al., 1994)

The stock *y, w; Sp/CyO; hsp-Cre (666-10) Sb/TM6B* has not been published yet (K. Basler).

en-Gal4 and *arm-Gal4* flies were kindly provided by K. Basler.

y, w; ap-Gal4 (Bloomington stock center)

y, w; act-Gal4/TM6By^{+} (Bloomington stock center)

pnr-Gal4 (*pnr*^{MD237}) (Bloomington Stock Center)

69B-Gal4 (Bloomington stock center)

puc^{lacZ} (Bloomington Stock Center)

Df(3)urd (Bloomington stock center)

EP(3)0929 (Bloomington stock center)

EP(X)1310 (Bloomington stock center)

cdc2^{E1-23} (Bloomington stock center)

UAS-AP-1sigma (this study)

UAS-CG30421 (this study)

UAS-falafel (this study)

UAS-falafel-HA (this study)

flfl^{1}*-flfl*^{5} alleles (this study)

EP screen

We made use of two new versions of an EP element called pDA530 and pDA543 (D. Nellen and K. Basler, personal communication). pDA530 and pDA543 drive expression of neighboring genes on both sides of their insertion site because of two sets of UAS (each set contains 10 copies of the UAS) pointing into opposite directions (Fig. 4). One of the two UAS sites is flanked by Cre recombinase target (loxP) sites to allow removal of this UAS site in flies expressing Cre

recombinase (Siegal and Hartl, 1996). The loxP sites are defective in pDA530, which was used during the first part of the screen (EP insertion numbers until EP12-118). pDA543, which was used in the second part of the screen, is identical to pDA530 except for the functional loxP sites. Transgenic flies containing an insertion of pDA530 or pDA543 were used as "starter lines", in which the EP insertion was mobilized in the presence of *P(Δ2-3)99B* transposase (Rorth, 1996). Males carrying novel EP insertions were subsequently crossed to *y, w; GMR-Gal4; Drac1^{N17}* or *y, w; sev-Gal4; Dcdc42^{N17}* virgin female flies exhibiting a rough eye phenotype. F1 progeny were scored under a dissecting microscope for modification of the rough eye phenotype. Lines that suppressed the original phenotype were retested at least once.

Identification of genes responsible for suppression phenotype

Plasmid rescue was done as described (Pirrotta, 1986) using EcoRI. For sequencing the isolated plasmids, we used primer P3' (5'-CGACGGGACCACCTTATGT TATTTCATCATG-3'). Flanking sequence was analyzed at the National Center for Biotechnology Information (NCBI) database (http://www.ncbi.nlm.nih.gov/blast) or the Berkeley *Drosophila* Genome Project (BDGP) database (http://www. fruitfly.org/blast/) using the Basic Local Alignment Search Tool (BLAST). Identification of the genes surrounding the P-element insertion was performed with the GeneSeen (Gadfly) (http://www.fruitfly.org/cgi-bin/annot/query) and Flybase (http://flybase.bio.indiana.edu/) databases.

A subset of the EP insertions was analyzed by Cre recombinase mediated excision of the UAS site, which is flanked by loxP (CRT) sites (Fig. 4). Briefly, flies containing the EP insertion and *hsp-Cre* were generated. These flies express Cre recombinase sufficiently in the absence of a heat-shock. Subsequently, these flies were crossed to balancer flies. F1 flies that carried EP-containing chromosomes and were phenotypically *yellow* were selected. These "single-headed" EP insertions, which lack the loxP-UAS-GAGA-yellow-ampR-loxP cassette (Fig. 4), were now assessed for their ability to suppress the *GMR-Gal4>UAS-Drac1^{N17}* or *sevGal4>UAS-Dcdc42^{N17}* phenotypes. This approach allowed us to identify the gene which is responsible for the suppression phenotype for the following EP insertions: EP22-048, EP29-104, EP31-149, EP32-120, EP33-059, EP36-054, EP39-161, EP47-082, EP53-065 (see Chap. 2.6.2., Tab. 4).

Isolation of *falafel* alleles

We performed two mutagenesis screens in which we selected mutated chromosomes carrying EP47-082 that either no longer showed the specific wing phenotype when crossed to 69B-Gal4 or were viable in combination with *act-Gal4*. For this purpose, EP47-082 males were fed 25 mM

EMS (ethylmethane-sulfonate) (Lewis, 1968) and subsequently crossed to *69B-Gal4* or *act-Gal4* virgins. 4000 F1 flies were screened for a reversion of the wing phenotype of *69B>EP47-082* flies. 32 selected flies were subsequently backcrossed to 69B, and three (*flfl*^{1}, *flfl*^{3} and *flfl*^{4}) showed germline transmission of the mutation. Seven revertants of the act-Gal4>EP47-082 lethality phenotype were backcrossed to act-Gal4, and two (*flfl*^{2} and *flfl*^{5}) showed germline transmission of the mutation. The molecular nature of the mutations was determined by amplifying genomic DNA of mutant flies by PCR and sequencing the PCR products. Promising mutations were verified by double peak analysis of the sequences using the Sequencher program (Gene Codes Corporation). We could identify mutations leading to amino acid substitutions (*flfl*^{1} and *flfl*^{2}) and premature stop codons (*flfl*^{3} and *flfl*^{4}). For the fifth EMS allele, the molecular nature is unknown.

Production of Germline Clones with the Autosomal FLP-DFS Technique

flfl alleles were recombined onto the FRT82B chromosome. Virgin females of the genotype y, w; FRT*flfl* / TM3, Ser, y+ were crossed to males of genotype hs70-FLP / Y; FRT P[ovoD1] / TM3, Sb. After a prelay at 25°C for one day, laid eggs were collected over a period of 24 h and incubated for another 36 h at 25°C. First instar larvae were then heat shocked for 1 hr at 37°C and put back to 25°C to complete development. 15-20 eclosed females of genotype y w / y w FLP; FRT *flfl* / FRT P [ovoD1] were mated with y, w / Y; FRT *flfl* / TM3, Ser, y+ males. Due to presence of the dominant female sterile mutation ovoD1 in the female germline, only germline clones homozygous for the *flfl* mutations can form eggs.

Cuticle preparations

Cuticle preparations of embryos were performed as described in Protocol 36.1 (Sullivan, 2000). Aged embryos were dechorinated in 50% bleach, rinsed and mounted in Hoyer's Mounting Medium. The medium was cured overnight at 60°C. These preparations were examined by dark-field microscopy.

Establishment of transgenic flies

To generate *UAS-falafel*, *UAS-ap-1sigma*, and *UAS-CG30421*, the ORFs coding for these genes were amplified by PCR from the full-length cDNA clones LD13350, GH27809, LD14109, respectively (obtained from Research Genetics, Huntsville, AL). The PCR products were ligated into the pUAST *Drosophila* transformation vector (Brand and Perrimon, 1993). Additionally, a haemoglutinin (HA) tag was added to Falafel to follow ist subcellular localization. To this end, the

PCR product of *flfl* cDNA was cloned into the pCRII-TOPO cloning vector (Invitrogen) and sequenced. Subsequently, the flfl cDNA was cloned upstream of the haemoglutinin (HA) tag into the NotI site of the *Drosophila* transformation vector pUAST-4HA to generate UAS-falafel-HA, which has a C-terminal HA-tag. The plasmid pUAST-4HA was provided by Isabel Hanson (MRC Human Genetics Unit, Edinbrgh, UK). It is a modified version of plasmid pUAST (Brand and Perrimon, 1993), which was generated by cloning four HA repeats into the XhoI site of pUAST (Isabel Hanson, personal communication). The resulting plasmids *UAS-falafel*, *UAS-falafel-HA, UAS-ap-1sigma*, and *UAS-CG30421* were subsequently used to generate transgenic flies by means of P element-mediated germline transformation as described (Basler et al., 1991). The constructs were injected into y, w embryos.

Immunohistochemistry and histology

Antibody staining was done using mouse anti-HA primary (1:1500, Boehringer Mannheim) and anti-mouse FITC secondary antibodies (1:500). Pictures were taken using a Leica SP2 confocal laser scanning microscope.

To detect β-galactosidase activity, embryos and third instar imaginal discs were fixed and subjected to a standard X-gal color reaction at 37°C.

RNAi interference in *Caenorhabditis elegans*

C. elegans strains were cultured at 20°C as described (Brenner, 1974). RNAi was performed by feeding worms with dsRNA-producing *E. coli* as described (Kamath et al., 2001) with the following modifications:

Five to ten L1 larvae were placed on the RNAi plates, and the P0 and F1 generations were inspected for embryonic phenotypes. Three millimolar IPTG was added to the agar to induce the expression of dsRNA.

Using the oligos oMZ51 (5'-TGTCTTGTCTGCACTGAAGC-3') and oMZ52 (5'-CGCCTTAAAACA CGTCTAGG-3'), or oMZ53 (5'-AAAAGGATCCAAACTCTAATTGTTTGGTCC-3') and oMZ54 (5'-AAAAAAGCTTGAAGTAGCGTTACAATGTCG-3'), two fragments of the *C.elegans falafel* ortholog (F41E6.4/SMK-1) were PCR amplified. The PCR products were cloned into a feeding vector (L4440) between two T7 promoters in inverted orientation and then transformed into a bacterial strain carrying IPTG-inducible expression of T7 polymerase (Timmons and Fire, 1998). The bacterial *E. coli* strain (HT115(DE3)) was used, which lacks double-strand-specific RNase III, improving the ability to produce RNAi phenotypes (Kamath et al., 2001).

The dsRNA-producing *E. coli* were fed to wildtype worms expressing the adherens junction marker JAM-1::GFP (Mohler et al., 1998). This allowed to follow embryogenesis under

fluorescent light microscope. To this end, animals were mounted on 3% agarose pads in M9 solution containing 15 mM NaN3. GFP expression was observed under fluorescent light illumination with a Leica DMRA microscope equipped with a cooled CCD camera (Hamamatsu ORCA-ER) controlled by the Openlab 3.0 software package (Improvision).

6. References

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7. A novel, evolutionary conserved protein phosphatase complex involved in cisplatin sensitivity (supplementary paper)

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A Novel, Evolutionarily Conserved Protein Phosphatase Complex Involved in Cisplatin Sensitivity*[S]

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Using a combination of tandem affinity purification tagging and mass spectrometry, we characterized a novel, evolutionarily conserved protein phosphatase 4 (PP4)-containing complex (PP4cs, protein phosphatase 4, cisplatin-sensitive complex) that plays a critical role in the eukaryotic DNA damage response. PP4cs is comprised of the catalytic subunit PP4C; a known regulatory subunit, PP4R2; and a novel protein that we termed PP4R3. The *Saccharomyces cerevisiae* PP4R3 ortholog Psy2 was identified previously in a screen for sensitivity to the DNA-damaging agent and anticancer drug cisplatin. We demonstrated that deletion of any of the PP4cs complex orthologs in *S. cerevisiae* elicited cisplatin hypersensitivity. Furthermore human PP4R3 complemented the yeast *psy2* deletion, and *Drosophila melanogaster* lacking functional PP4R3 (*flfl*) exhibited cisplatin hypersensitivity, suggesting a highly conserved role for PP4cs in DNA damage repair. Finally we found that PP4R3 may target PP4cs to the DNA damage repair machinery at least in part via an interaction with Rad53 (CHK2). *Molecular & Cellular Proteomics 4:1725–1740, 2005.*

Reversible protein phosphorylation is a highly conserved, essential regulatory mechanism involved in a host of cellular processes. Yet, while the phosphorylation of regulatory molecules by kinases has been studied intensively, their subsequent dephosphorylation is much less well understood. In eukaryotes, dephosphorylation on serine/threonine residues is effected by two distinct groups of functionally diverse phosphatases, the phosphoprotein M (represented by a sole member in higher eukaryotes, PP2C) and PPP[1] families (1). Within the much larger PPP class, a common catalytic domain (of 280 aa) is highly conserved, whereas the N and C termini are more divergent and further separate the PPP proteins into subfamilies. A number of distinct PPP subfamilies have thus been characterized (PP1, PP2A, PP2B, PP5, and PP7; Ref. 2) based on sequence homology, associated regulatory subunits, sensitivity to different types of chemical inhibitors, and metal ion requirements. A major PPP subfamily that plays a variety of critical roles in a multitude of physiological processes is the PP2A-type phosphatases, PP2AC (the catalytic subunit of PP2A; human gene names *PPP2CA* and *PPP2CB*), PP4C (gene name *PPP4C*, formerly known as PPX), and PP6C (PPP6C; Ref. 3).

PP2A often functions as a standard trimeric complex with a catalytic (C) subunit (encoded by two genes in mammals) associated with one of many regulatory (or B) subunits via one of two adaptor (A) molecules (4, 5). The regulatory and adaptor subunits are thought to confer substrate specificity to the complex (5).

In contrast to PP2A, the supramolecular architecture and subunit composition of PP4 multiprotein complexes remains largely unknown. Two mammalian PP4 regulatory subunits were previously identified (here termed PP4R1 and PP4R2, gene names *PPP4R1* and *PPP4R2*; Refs. 6 and 7). Although PP4R1 shares some sequence homology with the PP2A adaptor proteins (*PPP2R1A* and *PPP2R1B*), it does not bridge PP4C and PP4R2; PP4R1 and PP4R2 display mutually exclusive PP4C interactions (Refs. 6 and 7; and see below). Other PP4C-interacting partners have also been reported (*e.g.* Refs. 8 and 9), but whether these proteins represent *bona fide* regulatory subunits or phosphatase substrates and how these binding proteins may affect PP4 activity are unclear.

To gain a better understanding of the composition, function, and regulation of PP4, we systematically analyzed mammalian and yeast PP4C-interacting proteins. In doing so, we

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[1] The abbreviations used are: PPP, phosphoprotein phosphatase; TAP, tandem affinity purification; NTAP, N-terminal TAP; CTAP, C-terminal TAP; PP, protein phosphatase; PP4cs, protein phosphatase 4, cisplatin-sensitive; aa, amino acid(s); TEV, tobacco etch virus; HA, hemagglutinin; HEK, human embryonic kidney; HRP, horseradish peroxidase; Pol, polymerase; YPD, yeast-peptone-dextrose growth medium; MGC, mammalian gene collection.

were able to characterize several different mutually exclusive PP4C-containing complexes and identified a novel, evolutionarily conserved PP4C binding partner (which we termed PP4R3) that assembles into a complex with PP4C and PP4R2. Interestingly deletion of the yeast PP4R3 ortholog *PSY2* was demonstrated previously to elicit hypersensitivity to the DNA damage-inducing drug cisplatin (10). We found that deletion of the other components of the yeast PP4C·PP4R2·PP4R3 complex also yielded cisplatin hypersensitivity; therefore we termed this complex PP4cs (protein phosphatase 4, cisplatin-sensitive; see below).

Platinum-based anticancer agents such as cisplatin and carboplatin display a broad range of activities against solid tumors (11, 12). However, the response to platinum-induced DNA damage is not well understood; multiple intracellular signaling pathways are clearly involved in DNA repair (12). A common problem with cisplatin-based cancer therapies is resistance to cisplatin. A better understanding of the cellular processes and effectors involved in the response to cisplatin treatment may thus improve our ability to treat cancer.

EXPERIMENTAL PROCEDURES

Plasmids and Yeast Strains—pcDNA3-flagA was a kind gift from Dr. S. Morino, and pcDNA3-GST and the original pcDNA3–3HA were generous gifts from Dr. H. Imataka. pESC-URA was purchased from Stratagene, and the yeast two-hybrid vectors were described previously (13, 14). The yeast tandem affinity purification (TAP) tag strains as well as diploid deletion strains in the BY4743 background and haploid MATα strains in the BY4742 background were from Open Biosystems. The yeast two-hybrid strains pJ69-4A and pJ69-4α as well as the vectors pOAD and pOBD-2 were described previously (13, 14).

To generate mammalian TAP tag vectors for the production of N-terminal (NTAP) and C-terminal (CTAP) fusion proteins, the TAP sequences (from the versions with two immunoglobulin binding domains, a TEV site, and a calmodulin binding domain) from the *Schizosaccharomyces pombe* vectors pREP-NTAP or pFA6a-2xPA-CTAP (Ref. 15; kind gifts from Dr. K. Gould, Vanderbilt University) were amplified by PCR. For NTAP, the 5′ primer introduced a KpnI site, whereas the 3′ primer introduced sites for PmeI, AscI, PacI, and BamHI. The PCR product was digested with KpnI and BamHI and inserted into the KpnI and BamHI sites of pcDNA3 (Invitrogen). For CTAP, the 5′ primer added an XhoI site followed by sites for PmeI, AscI, and PacI, whereas the 3′ primer introduced an ApaI site. The PCR product was digested with XhoI and ApaI and inserted into the XhoI and ApaI sites of pcDNA3. Inserts were completely sequenced. Unique sites and polylinker sequence for pcDNA3-NTAP and pcDNA3-CTAP are shown in Supplemental Fig. 1 and at www.proteomecenter.org.

To generate pcDNA3-flag$_{new}$ and pcDNA3-EE (Glu-Glu), adapters encoding the sequences MDYKDDDDKAAS and MEYMPMEAAS, respectively, were cloned into the KpnI and PmeI sites of pcDNA3-NTAP (thereby replacing the NTAP cassette with the appropriate epitope tag). pcDNA3–3HA$_{new}$ was generated by amplifying the triple HA tag from the original pcDNA3-3HA (a kind gift from Dr. H. Imataka) and inserting this tag into the KpnI/PmeI sites of pcDNA3-NTAP. All epitopes and multiple cloning site were sequenced.

Coding sequences for proteins of interest were amplified by PCR using *Pfu* Ultra from yeast genomic DNA (all yeast ORFs), a cDNA library from HeLa cells (Stratagene; hTIP41, NM_152902; PP2ACα, NM_002715; PP2ACβ, NM_004156; PP4C, NM_002720; PP6C, NM_002721), a cDNA library from human placenta (Ambion; PP4R2, NM_174907), a pcDNA3-alpha4 (alpha4) construct kindly provided by Dr. K. Arndt (NM_001551), or clones from the mammalian gene collection (MGC) or IMAGE clones (KIAA2010, NM_032560; KIAA1387, NM_020463; TCP1, NM_030752; PP1C, NM_002708). All accession numbers in parentheses are from GenBank™. Inserts were cloned in-frame into appropriate vectors. 3′ and 5′ junctions of all inserts or entire inserts obtained through library amplification were sequenced.

Preparation of Extracts and Detection of Expression Levels—For stable cell pools, low passage number HEK293 cells (ATCC; CRL-1573) were transfected with Lipofectamine PLUS (Invitrogen) according to the manufacturer's instructions and selected with 750 μg/ml active G418 (Mediatech cellgro). Selection medium was changed every 2–3 days for ~14 days when a stable cell population was obtained. Expression was monitored, and cells were amplified further to generate ~10–20 150-mm plates per experiment.

Cells were washed three times in ice-cold PBS, harvested by scraping, and then centrifuged to remove excess PBS. TAP lysis buffer (10% glycerol, 50 mM Hepes-KOH, pH 8.0, 100 mM KCl, 2 mM EDTA, 0.1% Nonidet P-40, 2 mM DTT, 10 mM NaF, 0.25 mM $NaOVO_3$, and 50 mM β-glycerophosphate supplemented with 1× protease inhibitor mixture (Sigma catalog number P8340), 5 nM okadaic acid, and 5 nM calyculin A) was added (0.5–1 ml of lysis buffer/150-mm plate), and the mixture was incubated on ice for 30 min. Lysis was further enhanced by performing two freeze-thaw cycles. In preliminary experiments, varying concentrations of the specific serine/threonine phosphatase inhibitors were used; the interactions reported here were observed under all conditions.

Saccharomyces cerevisiae TAP tag strains (Open Biosystems) were grown to OD 0.8–1.0 and harvested by centrifugation. After one rinsing step in 20 mM Hepes, pH 7.5, 10 mM EDTA, cells were pelleted and flash frozen. Pellets were thawed in TAP lysis buffer using 1 ml of lysis buffer/g. Yeast were lysed by glass bead beating.

For the preparation of cleared extract from mammalian cells or yeast, debris were pelleted via centrifugation (15,000 rpm for 30 min at 4 °C), and protein concentration was determined by a Bradford-type assay (Bio-Rad). Expression levels of recombinant proteins in transfected mammalian cells or yeast strains were analyzed by separating 10–25 μg of total cell extract by SDS-PAGE followed by immunoblotting with normal rabbit serum (ICN Biomedicals Inc.; 1:2000) as the primary antibody and donkey anti-rabbit-horseradish peroxidase (HRP) (Amersham Biosciences; 1:5000) as a secondary antibody.

TAP Tag Purification—The purification strategy used here was largely the same as that described by Rigaut *et al.* (16) with minor modifications (see below) and adaptation for direct LC-MS analysis. More details may be found at www.proteomecenter.org.

Mammalian cells or yeast were lysed in TAP lysis buffer, and extracts were centrifuged to remove debris as described above. Mammalian TAP tag purifications were performed with extract from 5–20 15-cm plates of stably transfected HEK293 cells (roughly 40–150 mg of protein extract). Alternatively TAP purification was performed with extract from 1–2 liters of yeast grown to OD 0.8–1.0. Extracts were incubated with 100 μl of packed, prewashed IgG-Sepharose beads (Amersham Biosciences) for 4–6 h at 4 °C with gentle agitation. Beads were pelleted and washed three times with TAP lysis buffer and three times with TEV cleavage buffer (10 mM Hepes-KOH, pH 8.0, 150 mM NaCl, 0.1% Nonidet P-40, 0.5 mM EDTA, and 1 mM DTT). Beads were then resuspended in 300 μl of TEV lysis buffer containing 100–200 units of recombinant AcTEV (Invitrogen) and returned to incubation with gentle agitation at 4 °C for 10–16 h. After TEV cleavage, the IgG beads were pelleted, and the super-

natant was transferred to a fresh tube. IgG beads were rinsed three times with calmodulin binding buffer (10 mM Hepes-KOH, pH 8.0, 150 mM NaCl, 10 mM β-mercaptoethanol, 1 mM MgOAc, 1 mM imidazole, 0.1% Nonidet P-40, and 2 mM $CaCl_2$), and all washes were combined with the supernatant. $CaCl_2$ (5 μl of a 1 M stock) was added to the mixture, which was centrifuged once more and then transferred to a fresh tube containing 75 μl of packed calmodulin-Sepharose beads (Amersham Biosciences). Incubation was performed for 2–3 h at 4 °C with agitation. The slurry was transferred into empty Bio-spin columns (Bio-Rad), and the flow-through was removed through gentle air pressure. Two washes in calmodulin binding buffer (750 μl each) were performed followed by three washes in calmodulin rinsing buffer (50 mM ammonium bicarbonate, pH 8.3, 75 mM NaCl, 1 mM MgOAc, 1 mM imidazole, and 2 mM $CaCl_2$). The last drops of rinsing buffer were drained with gentle pressure, and Bio-spin columns were transferred to fresh tubes. Two elutions with 100 μl each of calmodulin elution buffer (50 mM ammonium bicarbonate, pH 8.3, and 25 mM EGTA, pH 8.0) were performed, and the Bio-spin column was transferred into another tube for two additional elutions.

Trypsin Digest and Preparation for Mass Spectrometry—Sequencing grade modified trypsin (Promega; 0.5–1 μg) was added directly to the eluate. Digestion was performed overnight at 37 °C. Following digestion, the sample was lyophilized and then resuspended in reversed-phase HPLC buffer A (20 μl; 0.4% AcOH, 0.005% heptafluorobutyric anhydride in H_2O). Prior to loading onto the reversed-phase column, the sample was centrifuged at 13,000 rpm for 10 min, and the supernatant was transferred to a fresh tube.

LC-MS/MS—Microcapillary reversed-phase columns (75-μm inner diameter, 363-μm outer diameter; Polymicro Technology) were cut to a final length of 15–20 cm, and spray tips were pulled in-house by hand. Columns were packed in-house (12 cm) with Magic C_{18} 100-Å, 5-μm silica particles (Michrom, catalog number PM5/61100/00) using a pressure bomb. Prior to loading the sample, columns were equilibrated in HPLC buffer A. Half of the sample was applied to the column using a pressure bomb and then washed off line in buffer A + 5% acetonitrile for 30–60 min. The LC column was then placed in front of a Finnigan LCQ mass spectrometer, programmed for data-dependent MS/MS acquisition (one survey scan, three MS/MS of the most abundant ions). After sequencing the same species three times, the mass ± 3 Da was placed on an exclusion list for 3 min. Peptides were eluted from the reversed-phase column using a multiphasic elution gradient (5–14% acetonitrile over 5 min, 14–40% over 60 min, and 40–80% over 10 min). The remaining half of the sample was then processed in the same manner. To prevent cross-contamination, each sample was processed on a freshly prepared reversed-phase column.

Data Analysis—Raw files generated by Xcalibur (Finnigan) were converted to the mzXML format (17), and combined runs (from the same sample) were searched using SEQUEST against the human International Protein Index (IPI) database, version 3.01, or against the *Saccharomyces* Genome Database (SGD) yeast ORF database (April 22, 2004 version). SEQUEST searches were performed without constraining for the number of tryptic termini, with a mass tolerance on the precursor ion of ±2, and methionine oxidation (+16) as a variable modification. SEQUEST html output was analyzed with INTERACT (18), PeptideProphet (19), and ProteinProphet (20) using the default parameters of each program. With the exception of Xcalibur and SEQUEST, all other software tools are open source and available from the Institute for Systems Biology (www.proteomecenter.org/software.php).

Non-radioactive Transcription/Translation Assays—Mixtures of plasmid DNA containing a T7 promoter (100–500 ng) were used to program 25 μl of a reticulocyte transcription/translation system containing Transcend tRNA (Promega) essentially according to the manufacturer's instructions. Translated proteins were subjected to SDS-PAGE and transferred to a nitrocellulose membrane. The membrane was blocked for 1 h in PBS + 0.5% Tween 20. Streptavidin-HRP (Amersham Biosciences; 1:10,000) was used to detect the newly synthesized proteins incorporating biotinylated lysine. After washing the membrane (3 × 5 min with PBS + 0.5% Tween 20; 3 × 5 min with water), chemiluminescence was performed. Immunoprecipitations were performed by diluting the TNT reaction with TAP lysis buffer and incubating with 5–10 μl of packed anti-HA beads (Roche Applied Science) or anti-FLAG resin (Sigma). After three washes with TAP lysis buffer, the sample was eluted directly in protein sample buffer lacking reducing agent by incubation for 30 min at 37 °C. Reducing agent was added, and the sample was boiled and loaded onto SDS-PAGE gels.

Cisplatin Sensitivity Assay in Drosophila—*flfl*^{2} (I57T) and *flfl*^{3} (premature stop codon at position 347) are ethyl methanesulfonate-induced alleles. Healthy heterozygous flies carrying either *flfl*^{2} or *flfl*^{3} over a marker chromosome (MKRS) were maintained on apple agar plates at 25 °C and allowed to lay eggs for 24 h. Agar plates were then changed and incubated for another 24 h at 25 °C. First instar larvae were collected and distributed to vials containing standard fly food into which the cisplatin solution was allowed to diffuse. Cisplatin powder (Alexis Biochemicals, Lausanne, Switzerland) was dissolved in 0.9% NaCl to obtain 0.4, 0.8, and 1.2 mM stock solutions. To test for putative undesirable effects of the MKRS marker chromosome, MKRS/+ flies were also tested for cisplatin sensitivity. The MKRS/+ as well as the control *flfl*^{2}/+ or *flfl*^{3}/+ flies showed no change in cisplatin sensitivity as compared with wild type flies.

RESULTS

Identification of Human PP4C-binding Proteins—TAP tagging and mass spectrometry, in which a protein complex of interest is purified in a two-step affinity enrichment process and its components are identified by the fragment ion spectra of selected tryptic peptides, have proven to be invaluable tools in the characterization of protein complexes (16, 21–23). As compared with single tag purification strategies, samples isolated by TAP tagging are significantly cleaner, thereby reducing the likelihood of false-positive identifications. Harsh washing conditions are also not necessary, allowing for recovery of native complexes. Another advantage of such approaches is that they are generic, making them particularly suited to the study of interaction networks in which binding partners for multiple proteins can be analyzed in parallel.

To gain insight into the supramolecular architecture of PP4-containing complexes, we tagged the catalytic subunit at its N terminus with a TAP tag, expressed this protein stably in HEK293 cells, and then processed the tagged protein along with its binding partners for purification (see "Experimental Procedures" and Fig. 1*A*). Eluted proteins were subjected to LC-MS/MS and identified via database searching followed by statistical analysis of the search results using a suite of software tools, including PeptideProphet and ProteinProphet (Refs. 19 and 20; see "Experimental Procedures"). To establish a list of background contaminant proteins, we analyzed cells stably expressing the TAP tag alone. In addition, proteins detected in numerous unrelated samples were flagged as dubious interactors and added to the contaminant list (see Supplemental Table I). For a list of PP4C-interacting pro-

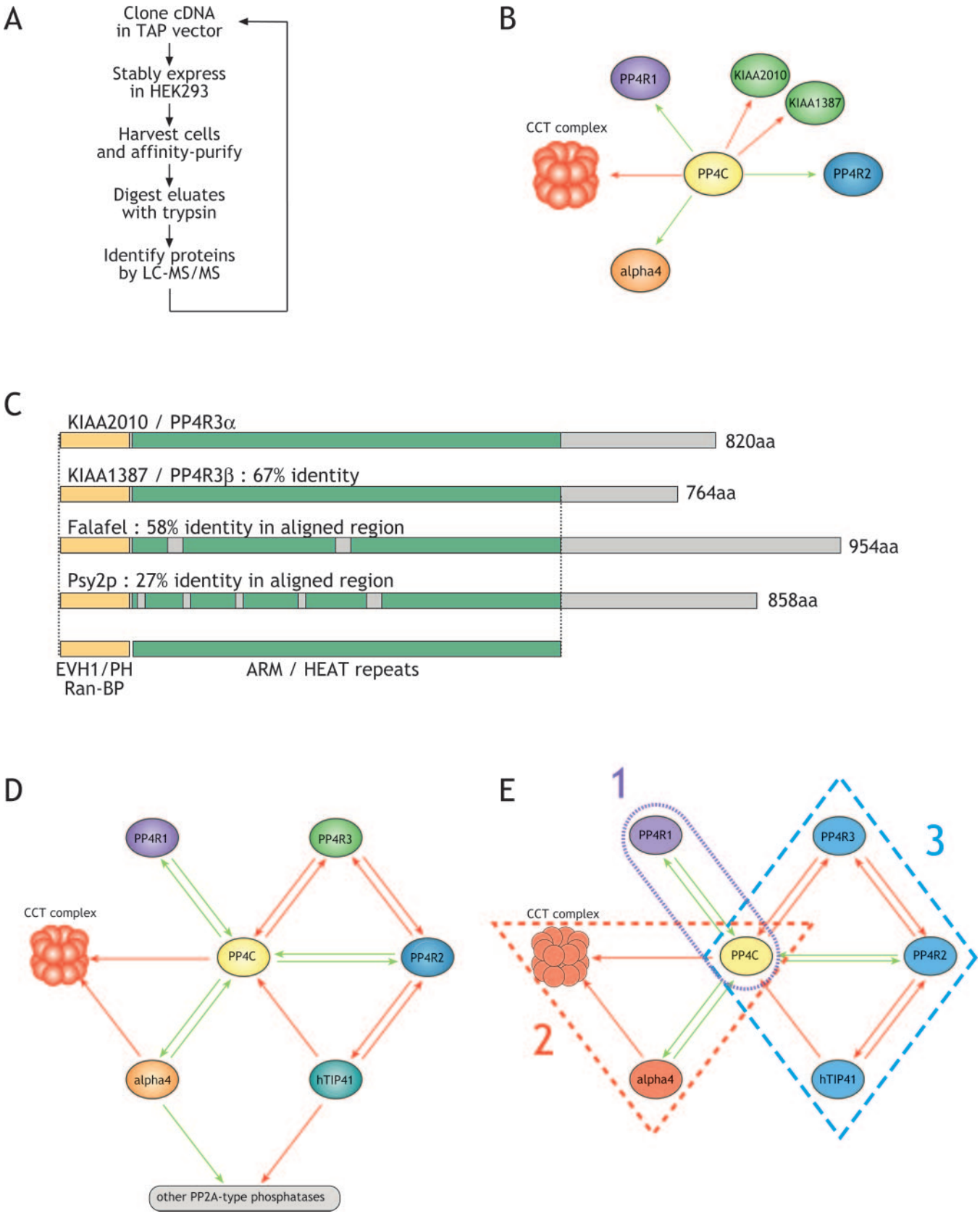


TABLE I
Human protein interactions detected by mass spectrometry

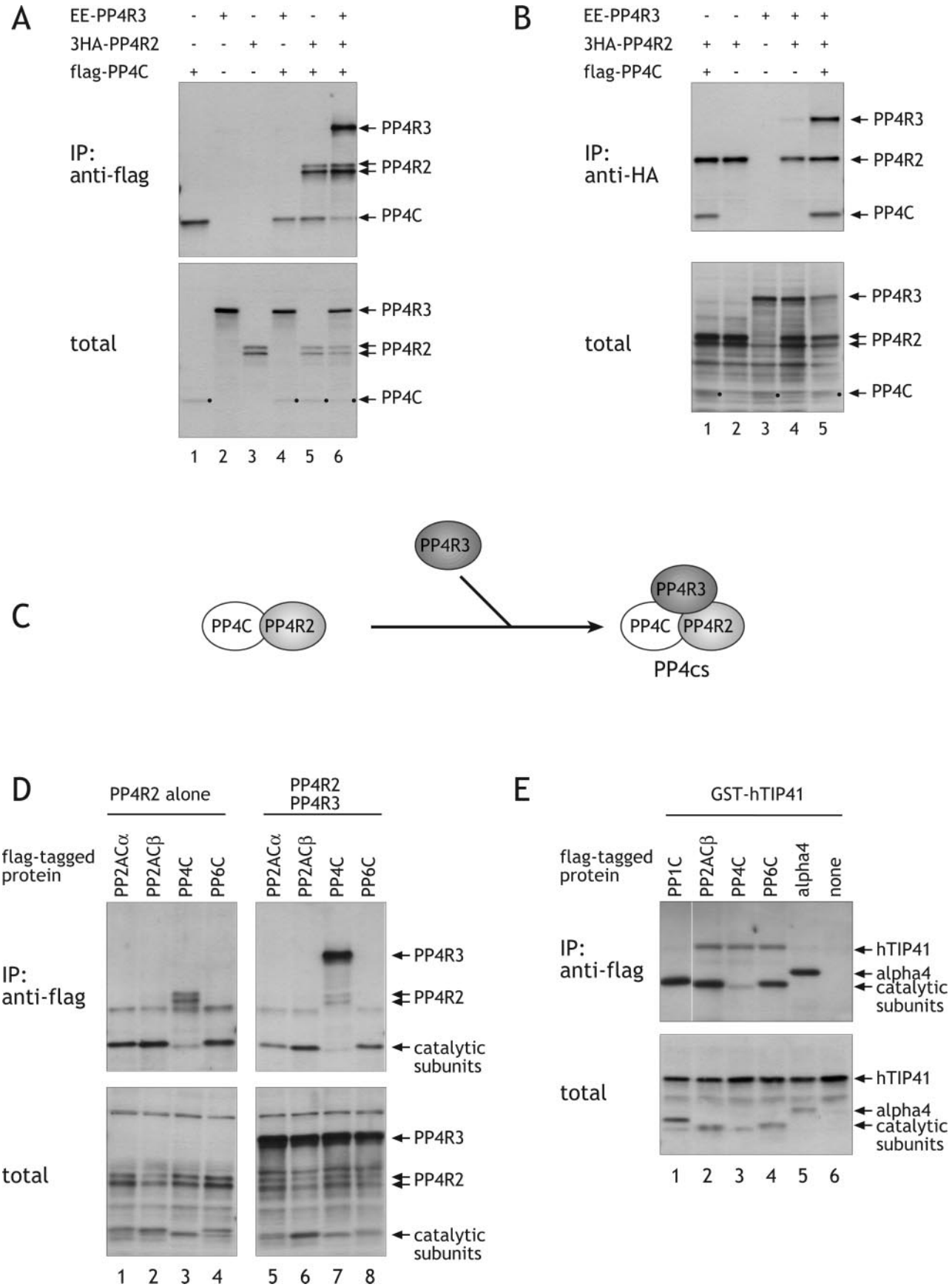
		Statistical data for interacting partners					
Tagged protein	Interactors	Accession	Protein Prophet p value	Unique peptides	Total peptides	Percent protein coverage	Coverage ratio
PP4C	IGBP1	NP_001542	1.00	10	16	33.6	0.85
	PP4R1	NP_005125	1.00	3	8	5.7	0.14
	PP4R2	NP_777567	1.00	11	20	42.2	1.07
	TCP1	NP_110379	1.00	13	24	29.8	0.76
	CCT2	NP_006422	1.00	15	22	34.4	0.87
	CCT3	NP_005989	1.00	5	11	14.9	0.38
	CCT4	NP_006421	1.00	7	15	22.2	0.56
	CCT5	NP_036205	1.00	15	30	35.5	0.90
	CCT6A	NP_001753	1.00	9	18	22.6	0.57
	CCT7	NP_006420	1.00	14	29	31.7	0.80
	CCT8	NP_006576	1.00	17	22	29.8	0.76
	KIAA2010/ PP4R3α	NP_115949	1.00	11	19	20.0	0.51
	KIAA1387/ PP4R3β	NP_065196	1.00	3	3	5.6	0.14
PP4R1	PP4C	NP_002711	1.00	14	31	40.1	1.70
IGBP1	TCP1	NP_110379	1.00	20	49	45.1	0.62
	CCT2	NP_006422	1.00	25	54	44.5	0.62
	CCT3	NP_005989	1.00	19	35	30.7	0.42
	CCT4	NP_006421	1.00	14	41	29.2	0.40
	CCT5	NP_036205	1.00	25	46	36.4	0.50
	CCT6A	NP_001753	1.00	19	42	35.2	0.49
	CCT7	NP_006420	1.00	17	32	35.4	0.49
	CCT8	NP_006576	1.00	24	50	39.6	0.55
	PP4C	NP_002711	1.00	1	1	6.2	0.09
	PP6C	NP_002712	0.98	1	2	6.2	0.09
	PP2AC	NP_002706	1.00	3	11	8.4	0.12
TCP1	CCT2	NP_006422	1.00	24	56	50.7	1.00
	CCT3	NP_005989	1.00	17	34	26.8	0.53
	CCT4	NP_006421	1.00	14	26	31.2	0.61
	CCT5	NP_036205	1.00	18	47	41.8	0.82
	CCT6A	NP_001753	1.00	17	39	35.2	0.69
	CCT7	NP_006420	1.00	18	39	31.7	0.62
	CCT8	NP_006576	1.00	19	25	29.6	0.58
PP4R2	PP4C	NP_002711	1.00	13	41	25.4	0.38
	KIAA2010/ PP4R3α	NP_115949	1.00	36	98	44.3	0.66
	KIAA1387/ PP4R3β	NP_065196	1.00	12	24	17.0	0.25
	MGC3794/ hTIP41	NP_690866	1.00	3	3	23.2	0.45
hTIP41	PP4C	NP_002711	0.94	1	1	6.5	0.13
	PP4R2	NP_777567	1.00	3	7	15.1	0.30
PP4R3α	PP4C	NP_002711	1.00	4	6	24.4	0.63
	PP4R2	NP_777567	1.00	16	28	49.6	1.28

teins see Table I. The data are also represented pictorially in Fig. 1*B*.

In addition to the recovery of the TAP-tagged "bait" protein itself (PP4C), we observed a number of peptides derived from the known PP4C regulatory subunits PP4R1 and PP4R2 (Table I). We also observed many peptides derived from alpha4 (*IGBP1*) previously demonstrated to interact directly with all of the PP2A-like phosphatase catalytic subunits (24, 25). Alpha4 is the ortholog of the yeast Tap42 protein, which links the target of rapamycin signaling pathway to PP2A-type phosphatases (26–28). However, the role of the PP2A-type phosphatases in rapamycin-sensitive signaling in mammals is not well understood.

Interestingly our analysis also identified all eight subunits of

FIG. 1. **Protein-protein interactions surrounding the mammalian PP4 catalytic subunit and identification of novel PP4C-interacting partners.** *A*, iterative TAP tag strategy to detect interacting proteins. *B*, proteins present in the TAP tag purification of the PP4 catalytic subunit. Only interactions detected in our TAP tag experiments are represented. *Green arrows* represent interactions that were also previously reported, whereas *red arrows* indicate novel interactions. *C*, PP4R3 is conserved throughout eukaryotic evolution. Shown is the alignment between KIAA2010, KIAA1387, Falafel, and Psy2p. *Green boxes* represent armadillo (*ARM*)/HEAT repeats, and the *yellow box* indicates a domain with homology to the Ran binding domain of RanBP1 as well as moderate homology with pleckstrin homology (*PH*) and EVH1 domains. *D*, PP4C interaction network. *Arrows* indicate directionality; bait → prey. hTIP41 and alpha4 were also found to be associated with the PP2A and PP6 phosphatases. *E*, PP4C appears to be a component of several different types of mutually exclusive complexes.



an ATP-dependent chaperonin, the TRiC/CCT complex. The TRiC/CCT complex was initially thought to be highly specialized for the folding and assembly of actin and tubulin, but recent work suggests that it participates in the folding and assembly of a broader range of substrates (23). The interaction with TRiC/CCT appears to be evolutionarily conserved as both Pph3 (the ortholog of PP4C) and Tap42 also co-precipitated with this complex in a large scale study (29). The functional significance of the TRiC/CCT complex interaction with PP4cat is unknown at this time.

A Novel Evolutionarily Conserved Protein Interacts with PP4C—Other than the known PP4C-interacting partners and the TRiC/CCT complex, only two additional (non-contaminant) proteins were detected in the TAP-PP4C purification: KIAA2010 and KIAA1387. The human KIAA2010 is 820 aa in length, and KIAA1387 is 764 aa. The two proteins share 67% sequence identity and 77% homology at the amino acid level (Fig. 1*C*). To be consistent with the nomenclature of the PP2A-type phosphatases, here we refer to KIAA2010 as PP4R3α and to KIAA1387 as PP4R3β (see below for further justification). PP4R3-like proteins are conserved throughout Eukaryotae: the *Drosophila* falafel (*flfl*) protein shares 58% identity with KIAA2010 at the amino acid level, and the *S. cerevisiae* Psy2 protein exhibits 27% identity with KIAA2010 over 699 amino acids (Fig. 1*C*).

The predicted two- and three-dimensional structure of the PP4R3 protein family indicates that a 115-aa fragment at the extreme N terminus shares extensive structural homology with a Ran binding domain found in RanBP1 (Fig. 1*C*). The predicted structure of this fragment is also reminiscent of pleckstrin homology domains. The Ran binding-like region is the most conserved portion of the protein across species, sharing 46% identity (60% similarity) at the amino acid level with yeast Psy2p and 87% identity (95% similarity) with *flfl*. Threading algorithms (30) indicate that the KIAA2010 and KIAA1387 proteins also possess a number of HEAT or armadillo repeats. Whether PP4R3 can interact with Ran is unknown at this time; we were unable to detect this interaction by co-immunoprecipitation following *in vitro* translation or co-transfection in 293 cells (data not shown).

Interestingly small interfering RNAs directed against KIAA1387 shorten mitotic transit time (31). Falafel (*flfl*) was first identified in a modular misexpression screen designed to identify novel dRac-specific signaling components; loss of *flfl* resulted in defects in dorsal closure, a phenomenon also associated with dRac deletion (Ref. 32; see below). Finally the yeast PP4R3 homolog was dubbed Psy2p based on hypersensitivity of deletants to the anticancer drug cisplatin (Ref. 10; see below).

Mammalian PP4 Module Interaction Network—To confirm the observed novel interactions and to better understand the supramolecular architecture of PP4C-containing complexes in mammals, we cloned the human alpha4, PP4R1 (*PPP4R1*), PP4R2 (*PPP4R2*), and PP4R3α (*KIAA2010*) ORFs into TAP tag vectors, established HEK293 cell lines stably expressing these tagged proteins, and repeated the purification and mass spectrometric identification process for each of these proteins and their interacting partners (Fig. 1, *A* and *D*, and Table I).

The PP4R1 pull-down yielded only the bait itself and PP4C. No peptides derived from alpha4, the TRiC/CCT complex, PP4reg2, or PP4reg3 were detected in these experiments (Table I). Alpha4 pull-downs yielded primarily TRiC/CCT complex proteins in addition to a single PP4C peptide and a few peptides from the other known alpha4 binding partners (PP6C, one peptide; and PP2AC, three peptides). In a PP4R2 pull-down, the TAP-tagged protein itself and PP4C were identified as well as many peptides from PP4R3α and PP4R3β. Thus, as with PP4C, PP4R2 appeared to be present in a complex with PP4R3α and/or PP4R3β. In addition to these proteins, the PP4R2 pull-down also yielded MGC3794, the human homolog of *S. cerevisiae* Tip41 (Tap42-interacting protein, molecular mass of 42 kDa), a protein involved in rapamycin signaling through phosphatase regulation (33, 34). We cloned the MGC3794 ORF (hereafter referred to as hTIP41) into the TAP tag vectors and repeated the pull-down/identification process with this fusion protein. PP4C and PP4R2 peptides (in addition to other phosphatase subunits, which will be reported elsewhere) were identified. This observation is intriguing because the yeast Tip41 protein was reported to interact with the yeast alpha4 homolog Tap42 and to prevent Tap42 from forming complexes with phosphatases (Ref. 33; see "Discussion"). Finally a pull-down of PP4R3α (KIAA2010) confirmed the interaction with PP4C and PP4R2. No additional proteins were identified in the PP4R3 pull-down.

Taken together, these data suggest that PP4C is present in several distinct complexes: 1) a binary complex with PP4R1, 2) a complex involving alpha4 and the chaperonin TRiC/CCT,

FIG. 2. **Assembly and specificity of the PP4C·PP4R2·PP4R3 complex.** *A* and *B*, epitope-tagged versions of PP4R3 (EE tag), PP4R2 (3HA tag), and PP4C (FLAG tag) were co-translated in a reticulocyte lysate containing biotinylated lysine (± denotes addition of the corresponding cDNA to the reaction). Following co-translation, reactions were either analyzed directly by SDS-PAGE (*bottom panels*) or incubated with an anti-FLAG Sepharose resin (*A*) or anti-HA Sepharose resin (*B*) prior to gel analysis. Proteins were transferred to nitrocellulose and visualized by incubation with streptavidin-HRP followed by chemiluminescence. *Black dots* denote the location of the weaker PP4C band. *C*, model depicting the preassembly of a binary complex between PP4C and PP4R2 prior to the stable association of PP4R3 into PP4cs. *D* and *E*, specific interactions of PP4R2 and PP4R3 (but not hTIP41) with PP4C. PP4R2 (3HA-tagged; *D*), PP4R2 + PP4R3 (EE-tagged; *D*), or hTIP41 (GST-tagged; *E*) were co-translated in a reticulocyte lysate with the indicated FLAG-tagged catalytic subunit. Following translation, samples were analyzed directly via SDS-PAGE (*bottom panels*) or immunoprecipitated as above using an anti-FLAG Sepharose resin (*top panels*). *IP*, immunoprecipitation.

and 3) a complex containing PP4R2 and PP4R3, which may also include hTIP41 (Fig. 1*E*). The PP4C·PP4R2·PP4R3 complex will be referred to throughout this study as PP4cs (see below). These results also demonstrate how an iterative TAP tagging/mass spectrometry approach may be used to characterize the components of individual multiprotein complexes.

Interaction of PP4R3 with PP4C Requires Preassembly of PP4C and PP4R2—We next confirmed that PP4C, PP4R2, and PP4R3α (KIAA2010) form a multimeric complex. Each protein was tagged at its N terminus with a different epitope tag to allow for detection and immunoprecipitation. Plasmid DNA coding for each of the tagged proteins was used to program a coupled transcription/translation (TNT) reticulocyte lysate containing biotinylated lysine (see "Experimental Procedures"). Following separation by SDS-PAGE and transfer to a nitrocellulose membrane, translation products were detected using streptavidin conjugated to HRP. When all three proteins were co-translated, both 3HA-tagged PP4R2 and Glu-Glu (EE)-tagged PP4R3 were co-immunoprecipitated with FLAG-tagged PP4C (Fig. 2A, *lane 6*). (The 3HA-PP4R2 constructs often yielded two products with slightly different electrophoretic mobilities. This is most likely because of alternative translation initiation start site usage, since the smaller product does not react with the anti-HA antibody.) Similarly a 3HA-PP4R2 protein pull-down (Fig. 2*B*, *lane 5*) co-precipitated both FLAG-PP4C and EE-PP4R3. Exclusion of PP4R3 from the TNT reaction had no apparent effect on the interaction between FLAG-PP4C and 3HA-PP4R2 (Fig. 2*A*, *lane 5*). Thus, binary complex formation between FLAG-PP4C and 3HA-PP4R2 is not dependent on PP4R3. Importantly, however, omission of 3HA-PP4R2 from the TNT mixture precluded co-immunoprecipitation of EE-PP4R3 with FLAG-PP4C (Fig. 2*A*, *lane 4*), suggesting that PP4R2 is necessary to mediate the interaction between PP4C and PP4R3. Consistent with this observation, a two-hybrid association between the putative yeast orthologs of PP4R2 (Ybl046w) and PP4R3 (Psy2p; Ynl201c) was reported previously in a large scale study (Ref. 35; also see below). However, a putative direct interaction between PP4R2 and PP4R3 in the TNT system is apparently not sufficient for the formation of a stable complex as omission of FLAG-PP4C from the TNT mixture prevented efficient co-precipitation of EE-PP4R3 with 3HA-PP4R2 (Fig. 2*B*, compare *lanes 4* and *5*). It thus appears that a binary complex between PP4C and PP4R2 must be assembled before PP4R3 can stably interact as illustrated in Fig. 2*C*. This situation differs from the assembly of PP2AC-containing trimeric complexes in that the adapter (A subunit, or *PPP2R1*) can efficiently co-precipitate either the catalytic or the regulatory B subunits (*e.g.* Ref. 36).

PP4R2 and PP4R3 Interact with PP4C but Not with the Related Phosphatases PP2AC or PP6C—Our TAP tagging data strongly suggested that the binding of PP4R3 and PP4R2 is specific to PP4C: no peptides derived from other phosphatases were recovered following TAP-mediated purification of PP4R2 or PP4R3. Similarly no PP4R2 or PP4R3 (KIAA2010 or KIAA1387) peptides were observed following TAP-mediated purification of PP2AC, PP6C, or PP1C (data not shown). To confirm these observations, we tested whether the two regulatory proteins can co-precipitate with the other mammalian PP2A-type phosphatases. The coding sequences for PP2ACα (*PPP2CA*), PP2ACβ (*PPP2CB*), and PP6C (*PPP6C*) were cloned into the pcDNA3-flag vector and co-translated in a TNT lysate with 3HA-PP4R2 alone (Fig. 2*D*, *lanes 1–4*) or 3HA-PP4R2 and EE-PP4R3 (*lanes 5–8*). All catalytic subunits were expressed (*bottom panel*), yet PP4R2 and PP4R3 were only co-precipitated on an anti-FLAG Sepharose resin when co-expressed with FLAG-PP4C (*lanes 3* and *7*). The interaction with PP4C is thus specific, and PP4R3 (KIAA2010) appears to be a *bona fide* PP4 regulatory subunit.

The specificity of the PP4cs intracomplex interactions contrasts with that of hTIP41, which interacted with all of the PP2A-like phosphatase catalytic subunits in our study. Consistent with this, GST-hTIP41 co-translated with the FLAG-tagged phosphatases in the TNT system was efficiently co-immunoprecipitated with FLAG-PP2ACβ, FLAG-PP4C, and FLAG-PP6C (Fig. 2*E*, *lanes 2–4*). However, the serine/threonine phosphatase PP1C (the PPP phosphatase most closely related to PP2A with 41% identity to PP2AC) was unable to precipitate hTIP41 (*lane 1*). In this respect, hTIP41 resembles the human alpha4 protein, which can establish interactions with all PP2A-type phosphatases (24). In contrast to a previous report documenting an interaction between the *S. cerevisiae* Tip41 and Tap42 proteins (33), hTIP41 was unable to co-precipitate FLAG-alpha4 (Fig. 2*E*, *lane 5*). Taken together, these results indicate that hTIP41 can interact with all of the PP2A-type phosphatase catalytic subunits and is therefore not a specific interacting partner for the PP4 complex.

Interaction among Yeast Orthologs of the PP4 Complex—The *S. cerevisiae* genome contains orthologs of the mammalian PP4C, PP4R2, and PP4R3 proteins. The protein sharing the most homology to PP4R3 in *S. cerevisiae* is Psy2 (Ynl201c; Fig. 1*C*). The yeast Pph3 protein (Ydr075w) is most closely related to PP4C, although the sequences of the other *S. cerevisiae* PP2A-type phosphatases (Sit4, Pph21, Pph22, and Ppg1) are also closely related. Although no yeast protein exhibits sequence homology to PP4R2 throughout its entire sequence, the Ybl046w ORF possesses a small stretch of homology in its N terminus. Large scale experiments have provided evidence for interactions between these yeast orthologs; a two-hybrid interaction was reported between Psy2 and Ybl046w (35), and complexes containing Pph3, Ybl046w, and Psy2 (along with other proteins) have been detected using pull-down/mass spectrometry approaches (29, 37). We thus set out to determine whether yeast Pph3-containing complexes were organized in a manner similar to mammalian PP4C-containing complexes and whether a PP4cs-like complex has been conserved throughout evolution. To this end, we obtained yeast strains expressing C-terminally TAP-

TABLE II
Yeast protein interactions detected by mass spectrometry

		Statistical data for interacting partners					
Tagged protein	Interactors	ORF name	Protein Prophet p value	Unique peptides	Total peptides	Percent protein coverage	Coverage ratio
Pph3	Ybl046w	YBL046w	1.00	42	133	53.1	1.29
	Psy2	YNL201c	1.00	63	241	61.2	1.49
	Spt5	YML010w	1.00	12	17	16.3	0.40
Ybl046w	Pph3	YDR075w	1.00	18	59	32.8	0.46
	Psy2	YNL201c	1.00	74	228	71.2	1.00
	Spt4	YGR063w	0.92	1	2	10.8	0.15
	Spt5	YML010w	1.00	33	62	36.3	0.51
Tip41	Ybl046w	YBL046w	0.98	1	3	3.4	0.05
	Psy2	YNL201c	1.00	4	5	7.1	0.10
Psy2	Pph3	YDR075w	1.00	21	45	52.3	0.77
	Ybl046w	YBL046w	1.00	44	147	66.7	0.99
	Tip41	YPR040w	1.00	2	4	11.2	0.17
	Spt4	YGR063c	1.00	2	4	25.5	0.38
	Spt5	YML010w	1.00	28	57	32.9	0.49
Spt5	Spt4	YGR063c	1.00	7	32	45.1	0.80
	Ybl046w	YBL046w	1.00	4	9	15.4	0.27
	Psy2	YNL201c	1.00	5	10	10.5	0.19
	Rpb2	YOR151c	1.00	8	8	8.1	0.14
	Rpb3	YIL021w	1.00	3	5	10.7	0.19
	Rpb4	YJL140w	1.00	2	2	11.8	0.21

tagged Pph3, Ybl046w, Psy2, and Tip41 proteins and used the same purification and mass spectrometry approach to identify interacting partners for these polypeptides. As shown in Table II and Fig. 3, interactions among the yeast proteins were extremely similar to those we observed for the human PP4cs proteins: Pph3, Ybl046w, and Psy2 all established reciprocal interactions. As in the mammalian system, Tip41p was also associated with this complex, although the coverage ratio suggests that it was present in lower amounts than the other components. We did not detect the TRiC/CCT complex or alpha4 in the Pph3-TAP pull-down; however, this may be due to the presence of the relatively large TAP tag at the C terminus of the protein (the mammalian PP4C was tagged at its N terminus) as both Pph3-FLAG and Tap42-FLAG were previously shown to co-precipitate with the TRiC/CCT complex (29).

Interestingly, we also detected the transcription elongation factor complex components Spt4 and Spt5 in TAP tag purifications of both Psy2 and Ybl046w (Spt5 was also detected previously as a Psy2 and Ybl046w interactor in a large scale experiment; Ref. 37). Conversely purification of TAP-tagged Spt5 yielded Spt4 and RNA Pol II subunits, as expected, in addition to several peptides for Psy2, Ybl046w, and Pph3. A putative role for PP4cs may thus be to target the Pph3 phosphatase to the transcription elongation machinery.

The Yeast PP4cs Complex Is Involved in the Response to Cisplatin-induced DNA Damage—A genome-wide scan for hypersensitivity to the DNA-damaging agents cisplatin and oxaliplatin (10) yielded three novel uncharacterized genes, including platinum sensitivity 2 (*PSY2*; YNL201c; Ref. 10). Interestingly *PPH3* deletion was also found to render cells hypersensitive to cisplatin treatment (10). Deletion of *PPH3* or *PSY2* was also reported to moderately increase the sensitivity of *S. cerevisiae* to methyl methanesulfonate (38). Because in mammalian cells PP4R2 is an obligatory partner for the association of PP4C and PP4R3, and Tip41 associates with PP4cs in yeast and mammals, we characterized the sensitivity of yeast lacking the *YBL046W* or *TIP41* (*YPR040W*) genes to cisplatin treatment. Diploid yeast strains were treated with cisplatin or vehicle alone, and the growth of yeast was monitored 36 h after plating onto fresh YPD agar plates. As expected from the large scale studies, deletion of *PPH3*, *PSY2*, or the polymerase ζ-associated protein *REV1*, rendered cells hypersensitive to cisplatin treatment (Fig. 3*C*). Importantly, and consistent with a role for the entire yeast PP4cs complex in cisplatin sensitivity, deletion of *YBL046W* also elicited cisplatin hypersensitivity. Interestingly, deletion of *TIP41* yielded an intermediate phenotype, exhibiting more sensitivity than a wild type strain yet less sensitivity than the *PSY2*, *PPH3*, or *YBL046W* deletants. Strains deleted for the *PPH3*-related *PPH22* or *PPZ2* phosphatases displayed cisplatin sensitivity similar to the parental strain in this screen. To control for spurious results due to secondary mutations, we repeated the experiment with a haploid deletion set and obtained identical results (Fig. 3*D*). Thus, the yeast PP4 complex containing Pph3, Ybl046w, and Psy2 plays a crucial role in viability following cisplatin-induced DNA damage. Tip41 may play a role as a modulator or facilitator perhaps by making the PP4 complex more available to substrate(s) following DNA damage treatment.

Because Psy2, Pph3, and Ybl046w interacted with Spt4 and Spt5 in the TAP tag pull-down experiments and Spt4 was implicated in the shutdown of Pol II-mediated transcription following DNA damage (possibly via dephosphorylation of Pol II Ser-5; Ref. 39), we reasoned that the involvement of the PP4cs proteins in cisplatin resistance may be effected

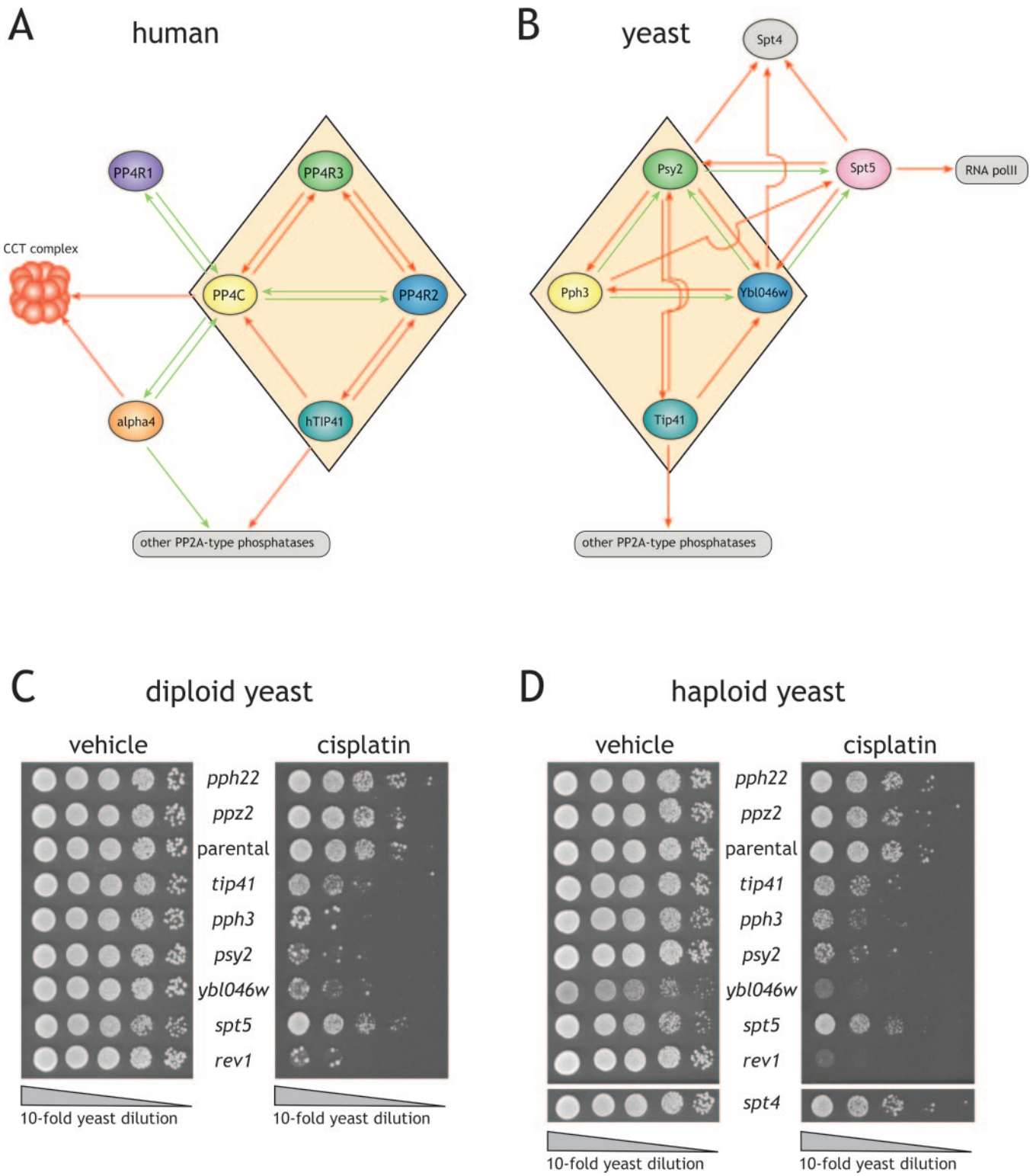


FIG. 3. **Comparison of yeast and human PP4C interaction maps and cisplatin sensitivity of individual yeast deletion strains.** *A* and *B*, schematic organization of the multiprotein complexes recovered and identified by TAP tagging and mass spectrometry. Human data are also shown in Fig. 1 and Table I; yeast data are extracted from Table II. *Arrows* denote directionality of the interaction (bait → prey) and are *color-coded* as in Fig. 1. *Colored nodes* represent proteins used as bait in the TAP tagging procedure, whereas *gray nodes* represent proteins that were not analyzed further. The *beige diamond* encompasses the PP4C·PP4R2·PP4R3·hTip41 complex conserved from yeast to mammals. *C* and *D*, cisplatin sensitivity of yeast strains harboring deletions for PP4cs complex components. Diploid (*C*) or haploid (*D*) *S. cerevisiae*

through *SPT4* and *SPT5*. Therefore, homozygous diploid *SPT5* deletant and haploid *SPT4* and *SPT5* deletion strains were tested for cisplatin sensitivity. In the absence of cisplatin, both strains grew slightly slower than the parental strain. However, none of the *SPT4/5* strains exhibited significant hypersensitization to cisplatin (Fig. 3, *C* and *D*). Thus, *SPT4* and *SPT5* are not likely to be key mediators of the cisplatin sensitivity caused by PP4cs deletion.

Psy2 Interacts with Rad53—In an attempt to elucidate how PP4cs and Tip41 are connected to the DNA damage repair machinery, we turned to a yeast two-hybrid strategy. Psy2 was previously reported to interact with Rad53 in the yeast two-hybrid assay (Rad53 was used as bait; Ref. 13). We attempted to verify this interaction using the reverse configuration with Rad53 cloned into a prey vector and Psy2 expressed from a bait vector. In addition, we tested pairwise interactions among all of the components of our yeast PP4 complex and explored putative interactions with other components of the DNA damage machinery. A total of 31 nonredundant prey-containing yeast strains were mated in duplicate to strains expressing Psy2, Ybl046w, Pph3, Tip41, and Rad53 from the bait vector. Although a previous study utilized Rad53 as bait, our own bait strains harboring Rad53 mated inefficiently, and two-hybrid interactions could not be tested for this strain. The other bait strains were tested in a 96-well format, and the growth of colonies on media lacking histidine was monitored. By plating the yeast on selection media containing varying amounts of 3-aminotriazole (a stoichiometric inhibitor of His3p activity), background levels could be efficiently controlled such that growth of negative controls (*e.g.* vector alone) served as a base line to score positives. As previously reported, bidirectional interactions were detected between Psy2 and Ybl046w (Fig. 4, *A* and *B*). In addition, Tip41 (bait) interacted with Pph3 (prey), Pph21, Pph22 (Fig. 4*A*), Sit4, and Ppg1 (not shown). We also detected a previously unreported bidirectional interaction between Pph3 and Ybl046w. Consistent with our TAP tag data, Psy2 strongly interacted with Spt4 and Spt5 in the two-hybrid assay. Finally a strong signal was also observed between Psy2 (bait) and Rad53 (prey). No signal was detected for Rad53 with any of the other baits tested. Additionally no signal was detected between any of the baits tested and strains containing prey vectors expressing other proteins involved in DNA damage (not shown). These data suggest that the yeast PP4cs complex is connected to the DNA damage machinery via an interaction between Psy2 (PP4R3) and Rad53.

PP4R3 Is a Functional Homolog of Psy2—To determine whether the yeast and human PP4cs are functionally equivalent in the cisplatin sensitivity assay, we reintroduced a FLAG-tagged galactose-inducible Psy2 or mammalian PP4R3 (or vector alone) into the *psy2*Δ diploid strain. We then performed cisplatin sensitivity assays as above. As expected, the *psy2*Δ strain (transformed with vector alone) was much more sensitive to cisplatin treatment than the parental strain (transformed with vector alone; Fig. 5*A*), although the magnitude of this effect was less pronounced on the synthetic defined media used for this assay than on YPD media as used in Fig. 4. *psy2*Δ strains transformed with FLAG-Psy2 (two independent transformants tested) exhibited a level of cisplatin sensitivity similar to the parental strain (Fig. 5*A*). Strikingly expression of FLAG-tagged mammalian PP4R3 also reverted the cisplatin hypersensitivity of a *psy2*Δ strain, indicating that Psy2 and PP4R3 are functionally equivalent in mediating resistance to cisplatin.

To determine whether human PP4R3 can establish the same two-hybrid contacts with the yeast prey proteins as Psy2, we mated a yeast bait strain expressing human PP4R3 to our prey array. Although we did not detect interactions with Ybl046w, Spt4, or Spt5, PP4R3 strongly interacted with yeast Rad53 (this was, in fact, the only interaction we detected; Fig. 5*B*). It thus appears that an interaction between PP4R3/Psy2 and the DNA damage machinery has been evolutionarily conserved.

Cisplatin Hypersensitivity of PP4R3 Mutants in Higher Eukaryotes—To investigate whether a reduction in PP4R3 activity renders a multicellular organism hypersensitive to cisplatin, we assayed animals harboring mutations in the *Drosophila* falafel (*flfl*) gene, which encodes the fly homolog of the PP4R3/Psy2 protein (Fig. 1*C*). Two ethyl methanesulfonate-induced mutant alleles, $flfl^2$ and $flfl^3$ (see "Experimental Procedures"), were identified in a reversion assay for an enhancer and promoter-mediated *flfl* gain-of-function phenotype.[2] Although flies homozygous for either mutation ($flfl^2/flfl^2$ or $flfl^3/flfl^3$) were inviable, 30% of animals possessing the heteroallelic combination $flfl^2/flfl^3$ reached adulthood and displayed a statistically significant reduction in size as compared with their wild type counterparts (weight reduction of ~23% in females and ~12% in males).

Mutant first instar $flfl^2/flfl^3$ larvae were placed onto standard fly food supplemented with increasing concentrations of cisplatin (see "Experimental Procedures"), and emerging adult flies were counted 3 days after eclosion. (The survival of mutant $flfl^2/flfl^3$ animals is compromised even in the absence of cisplatin, so these data were normalized to the survival of *flfl* mutants in the absence of the drug.) Survival curves for $flfl^2/flfl^3$ mutants and control animals grown on increasing

[2] M. Zarske and E. Hafen, manuscript in preparation.

deletion mutants were grown to OD ~ 0.4 and then treated with 1 mM cisplatin (or DMSO vehicle alone) for 4 h. Serial 10-fold dilutions were plated onto YPD, and growth was monitored 36 h post-treatment. Pph22 is a yeast homolog of the PP2A catalytic subunit, whereas Ppz2 is a homolog of the human PP1 catalytic subunit. *rev1*Δ was used here as a positive control.

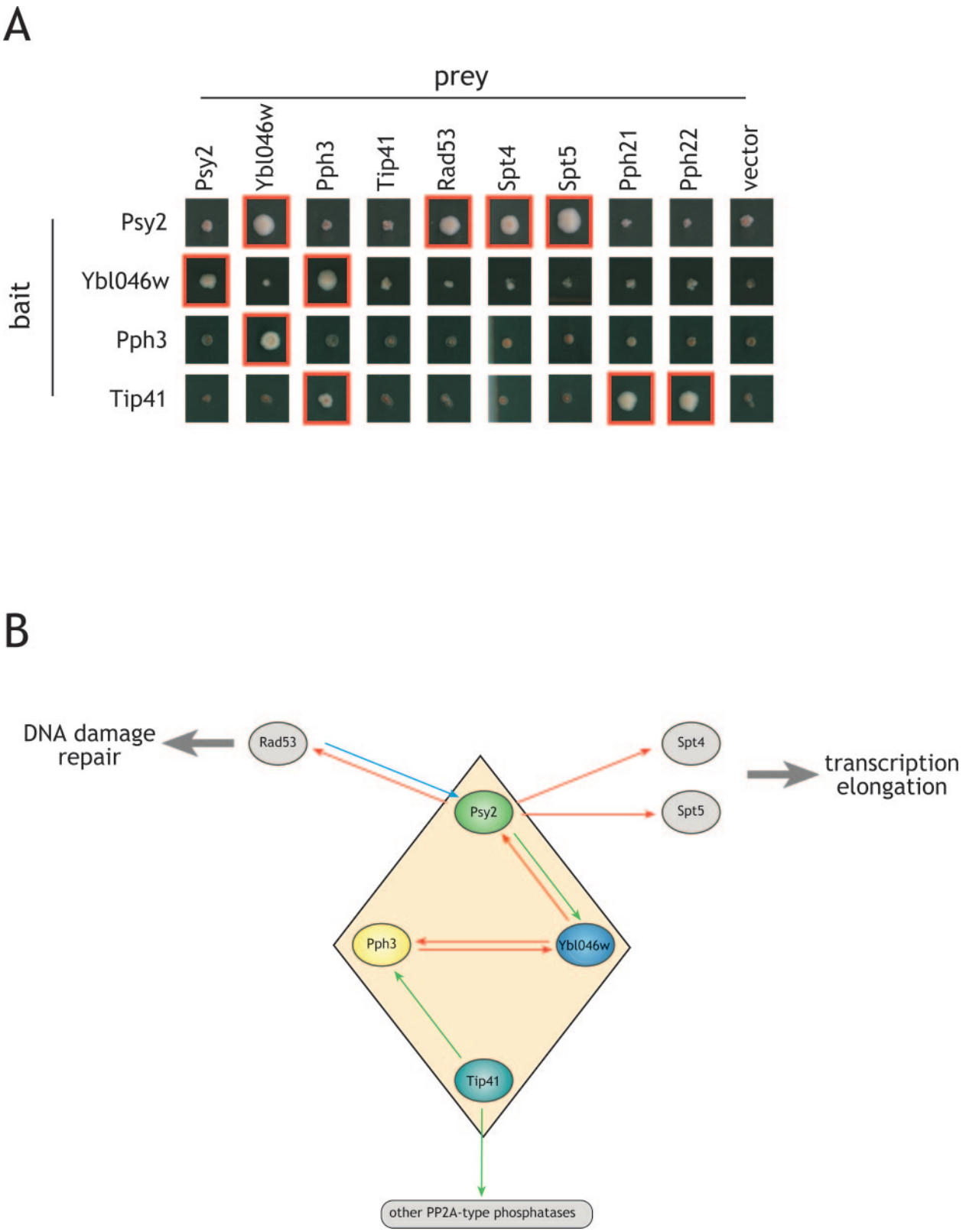


FIG. 4. **Psy2p interacts with Rad53p.** Coding sequences for Psy2p, Ybl046w, Pph3p, and Tip41p were cloned into the yeast two-hybrid bait vector pOBD-2, and the constructs were transfected into the pJ69-4a strain. A miniarray of "prey" in pOAD/pJ69-4α, extracted from a larger collection described previously (13), was supplemented with a collection of cDNAs coding for Psy2, Ybl046w, Pph3, Tip41, and Rad53 in pOAD/pJ69-4α and generated in parallel to the bait. The final prey array consisted of the following proteins: Spt4, Rad6, Rev7, Mrc1, Ybl046w,

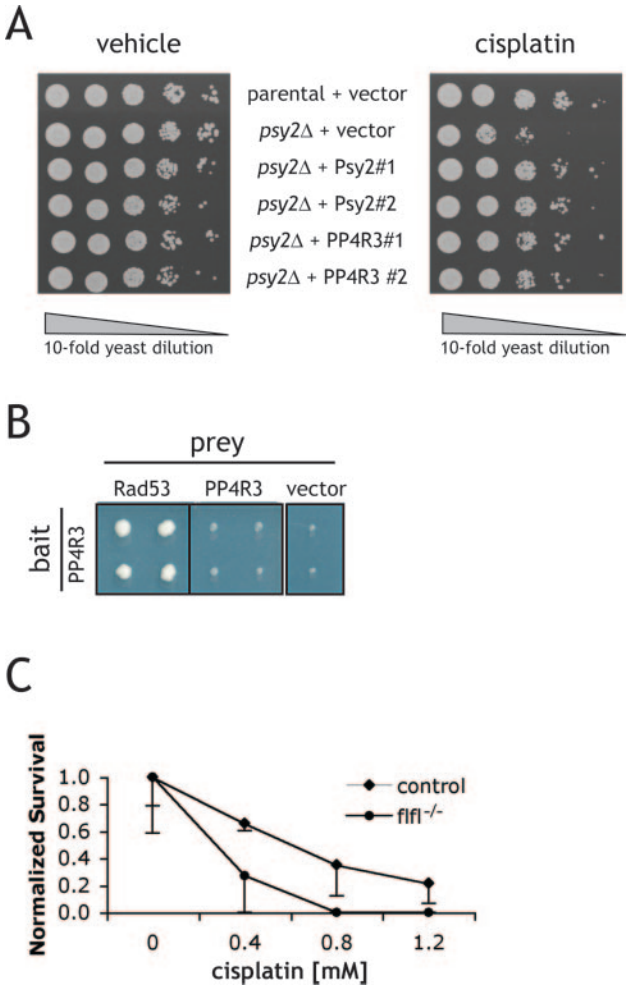


FIG. 5. **PP4R3 from higher eukaryotes is involved in cisplatin sensitivity.** *A*, mammalian PP4R3 functionally replaces *PSY2* in the cisplatin sensitivity assay. Yeast Psy2 or mammalian PP4R3 cDNAs were cloned into the galactose-inducible pESC-ura vector and transformed into the diploid yeast strain lacking *PSY2*. Individual clones were selected. Expression of the recombinant cDNAs was driven through growth on galactose for 8 h prior to cisplatin treatment (4 h). A serial 10-fold dilution series was plated onto minimal medium lacking uracil but containing galactose to maintain expression. Growth was monitored 48 h post-treatment. *B*, mammalian PP4R3 interacts with yeast Rad53. A yeast two-hybrid assay using human PP4R3 as bait was performed as in Fig. 5 in duplicate. *C*, flies lacking functional PP4R3 (*flfl*) are hypersensitive to cisplatin. Survival of *flfl* heterozygous (control, MKRS/$flfl^2$, or MKRS/$flfl^3$) and $flfl^2/flfl^3$ heteroallelic mutant female flies following cisplatin treatment. Numbers of surviving flies at various concentrations of cisplatin were normalized to the numbers of untreated animals. Similar results were obtained with male flies. Experiments were performed in triplicate at all concentrations with ~100 flies per concentration.

concentrations of cisplatin are shown in Fig. 5*C*. Female $flfl^2/flfl^3$ mutant flies displayed reduced survival at 0.4 μM cisplatin compared with wild type flies (Fig. 5*C*). No $flfl^2/flfl^3$ individuals survived when exposed to 0.8 or 1.2 μM cisplatin, whereas some wild type animals emerged (identical results were observed with male *flfl* mutant animals; data not shown). These results suggest that falafel (PP4reg3) function in higher eukaryotes is required for resistance to cisplatin treatment and provide further evidence for a critical and evolutionarily conserved role of this PP4C-interacting partner in the response to DNA damage.

DISCUSSION

Here, using an iterative TAP tagging/mass spectrometry approach, we report that the mammalian PP4 catalytic subunit is a component of several different mutually exclusive subcomplexes: one containing PP4R1, another containing alpha4 and the TRiC/CCT complex, and a third (PP4cs) containing PP4R2 and a novel protein that we termed PP4R3. The PP4cs complex is evolutionarily conserved and in yeast is composed of the Pph3, Ybl046w, and Psy2 proteins. The conservation in *S. cerevisiae* suggested a role for PP4cs in resistance to cytotoxic agents: genome-wide screens of the yeast deletion collection with the DNA-damaging agents cisplatin, oxaliplatin, mitomycin C, and methyl methanesulfate have highlighted the importance of both *PPH3* and *PSY2* in survival following exposure to these agents (10, 38). We demonstrated that yeast strains deleted for *YBL046W* (PP4R2) are hypersensitive to cisplatin to the same extent as strains deleted for either *PPH3* or *PSY2*. The mammalian ortholog of Psy2, PP4R3, could substitute for the yeast protein in cisplatin sensitivity assays, suggesting that the mammalian complex plays a similar role. Furthermore in *Drosophila*, mutation of the falafel gene, the ortholog of Psy2, led to increased cisplatin sensitivity.

How might the PP4C·PP4R2·PP4R3 complex be linked to the DNA damage response? A previous yeast two-hybrid screen for Rad53-interacting partners yielded Psy2 (13). We confirmed this observation and demonstrated that the interaction is independent of Rad53 kinase activity (data not shown). Rad53 (or CHK2 in humans) is a critical component in a DNA damage checkpoint response conserved from yeast to humans (human ortholog CHK2; Refs. 40 and 41). We were not able to detect Rad53-Psy2 or human CHK2-PP4R3 interactions by pull-down assay, suggesting that these interactions are transient or easily disrupted through our purification protocol or that the interactions may be substoichiometric.

Rad18, Rrd1, Rad10, Rad2, Pho85, Ppg1, Luc7, Pph3, Rrd2, Tip41, Pph22, Pph21, Ynr069c, Ahc1, Hol1, Psy2, Rad53, Rev1, Rad1, Spt5, Rad26, Rad9, Rev3, Mec1, Sit4, and Mrp144. Bait and prey strains were mated, and growth on selective medium (lacking histidine and containing various amounts of 3-aminotriazole) was monitored. *A*, representative results for each mating. Positive results are *framed* in *red*. *B*, summary of the interactions detected by the yeast two-hybrid method in this and other studies. Proteins used as bait are denoted by *colored nodes*. *Red arrows* indicate novel interactions reported for the first time in this study; *green arrows* indicate interactions detected in this study as in previous reports; and the *blue arrow* represents an interaction reported in the literature but not tested here.

Other links between PP4cs and DNA damage also exist. Yeast PP4cs associates with Spt4 and Spt5, transcription elongation factor proteins linked to DNA damage-induced Pol II dephosphorylation at Ser-5, and subsequent transcriptional shutdown (39). However, deletion of Spt4 or Spt5 did not result in hypersensitivity to cisplatin, indicating that the PP4cs-Spt4-Spt5 interaction is not sufficient to explain the cisplatin sensitivity phenotype.

Psy2 was also reported previously to genetically and physically interact with Wss1 and Tof1, two proteins presumed to play a role in the stabilization of stalled replication forks (42). In human cells, additional links between PP4C and/or the PP4cs complex and cisplatin-induced DNA damage may exist. For instance, cisplatin-induced NF-κB activation appears to be mediated, at least in part, by PP4C-mediated dephosphorylation of NF-κB p65 at Thr-435 (43). The precise contribution of each of these pathways to the cisplatin hypersensitivity phenotype of the PP4cs deletants remains to be assessed.

Cisplatin and related platinum compounds such as carboplatin are effective against many types of solid tumors, including cervical, ovarian, head-and-neck, and non-small cell lung cancers (for a review, see Ref. 44). Cisplatin analogs have also proven particularly effective against testicular cancer for which the overall cure rate exceeds 90% (for a review, see Ref. 12). However, intrinsic or acquired resistance to cisplatin analogs is frequently encountered, significantly limiting the application of this class of drugs (12). Resistance may be attributed to several factors, including decreased drug accumulation, increased cellular detoxification, enhanced DNA repair, tolerance to platinum-induced DNA damage, and alterations in signal transduction pathways (11). Cisplatin resistance is thus a major hurdle in cancer therapies, and a primary goal in designing new platinum-based anticancer agents has been to circumvent this problem (45).

The sensitization to cisplatin of *S. cerevisiae* deleted for any of the PP4cs members suggests that inactivation of the human PP4cs components during cisplatin treatment may sensitize cisplatin-resistant tumor cells to the drug. In support of this possibility, two different cell lines selected for cisplatin resistance through long term cisplatin treatment demonstrated a striking reversal to a sensitive phenotype when co-treated with low doses of demethylcantharidin or okadaic acid, two PP2A inhibitors (46, 47). PP4C is also sensitive to these compounds (48) and was likely inhibited in these studies. Novel platinum-based compounds comprised of two moieties, the platinum group and demethylcantharidin, elicited sensitivity of these cisplatin-resistant cell lines only when the compounds exhibited activity toward PP2A (46, 47). Based on the shared sensitivity of PP2AC and PP4C to cantharidin-based compounds, PP4C is also likely targeted by these compounds. A drug that targets the assembly of PP4cs, perhaps by preventing PP4R2 from binding to PP4C or PP4R3, could thus potentially synergize with cisplatin while having fewer side effects than drugs inhibiting all PP2A-type subunits.

Finally a different type of anticancer compound, rapamycin, was also demonstrated to enhance cisplatin sensitivity in cell and animal models (*e.g.* Ref. 49). Rapamycin actions are conserved from yeast to humans and appear to involve modulation of PP2A-type phosphatase activity through Tap42 (alpha4) and Tip41 (at least in yeast). Rapamycin analogs are used in the clinic to prevent graft rejection and as anticancer agents (for reviews, see Refs. 50 and 51). Recently the synergy between cisplatin and rapamycin was demonstrated to be attributable to changes in the levels of the p21 protein because of a modest general decrease in translation brought about by rapamycin (52). Whether PP4cs plays a role in the control of translation and the regulation of p21 levels remains to be investigated.

Further delineation of the interacting partners and supramolecular architecture of the PP2A-type phosphatases should greatly assist us in deciphering the roles that each of these important proteins plays in various cellular processes. Given that these enzymes play critical roles in DNA repair and tumor promotion (4), a more intimate understanding of their regulation and function should provide us with greater treatment options for various types of cancers.

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